

IVth European Cell Biology Congress

ABSTRACTS

Prague, June 26th - July 1st 1994.

P-1 TRANSMISSION OF INTRACELLULAR CYTOSKELETAL ORDER TO SUPRACELLULAR TISSUE ORGANIZATION

Werner W. Franke

Abstract not received

CELL TRANSFORMATION

J. Svoboda

P-2 Institute of Molecular Genetics,
Academy of Sciences of the Czech
Republic, Prague, Czech Republic

The nature of cell transformation has been attacked from different points of view but ultimately the somatic cell mutation and virus-mediated transformation hypotheses have predominated. Despite the initial antagonism between both hypotheses, the detailed study of acutely transforming retroviruses provided conclusive evidence that they acquired oncogenes which represent specifically modified cell genes involved in signal transduction, transcriptional regulation and differentiation. Using the established methodology, a number of non-viral oncogenes were characterized and tumour suppressor genes having distinct anti-tumour activity were discovered. These genes have an important control activity which involves also the cell cycle whose deregulation plays a crucial role in cell transformation. The genesis and regulation of an oncogene and a complex role of its normal cell counterpart - proto-oncogene will be illustrated on the oncogene *v-src* and proto-oncogene *c-src*. Signalling pathways related to selected oncogenes and proto-oncogenes will be designed and involvement of deregulation of the cell cycle and epigenetic gene modification discussed.

P-3 GENETIC CONTROL OF EUKARYOTIC DEVELOPMENT

F. C. Kafatos

Abstract not received

MICROTUBULE DEPENDENT ORGANIZATION
OF CYTOPLASMIC MEMBRANE TRAFFIC

P-4 Thomas E. Kreis

Department of Cell Biology, University
CH-1211 Geneva, Switzerland

Membrane traffic and the spatial arrangement of cytoplasmic, membrane-bounded organelles depends on microtubules. Microtubule-based motors and cytoplasmic linker proteins (CLIPS) regulate the interactions of membranous organelles with microtubules. These interactions may lead to the anchoring of organelles to a specific region of the cytoplasm, or to their motor protein driven movement along microtubules. Using *in vitro* binding assays we have analyzed the cytosol dependent binding of a variety of cellular organelles to microtubules. These interactions depend on different organelle specific cytosolic proteins and membrane associated receptors. We have identified and characterized CLIP-60 and CLIP-170 on the molecular level, proteins which bind TGN-derived carrier vesicles and endosomal vesicles, respectively, to microtubules. These CLIPS may represent organelle specific capturing devices that establish an initial contact between an organelle and a microtubule, before motor-dependent movement occurs.

P - 5

A NOVEL LYSOSOMAL COMPARTMENT ENGAGED IN ANTIGEN PRESENTATION
Hans J. Geuze
Laboratory of Cell Biology, Utrecht University School of Medicine, The Netherlands.

Major histocompatibility class II (MHC-II) molecules bind peptides which are proteolytically derived from exogenous antigens and present these to CD4 positive T-cells. MHC-II is a heterodimer of α and β chains, which assemble in the ER with the invariant chain (I-chain). Association of I-chain with MHC-II prevents the binding of endogenous peptides present in the ER lumen to nascent MHC-II while signals in the cytoplasmic tail of the I-chain are essential for targeting of newly synthesized MHC-II/I-chain complexes to an endocytic compartment where binding with peptide can occur (for a review see 1).

Using immunogold labeling of ultrathin cryosections we have localized MHC-II, I-chain and several organelle markers in a variety of antigen presenting cells. In human B-cells we found that the majority of the intracellular MHC-II molecules resides in a compartment (called the MHC-II-enriched compartment, MiIC) containing internal vesicles and membrane sheets and sharing several characteristics with lysosomes (2). MiIC's were also numerous in the dendritic cells of the spleen and in Langerhans cells.

In combined biochemical and morphological studies on macrophages we show that presentation of peptides derived from endocytosed protein was most efficient when the peptides were produced in lysosomes. Moreover we found that membranes of dense Percoll fractions enriched in MiIC's contained MHC-II/peptide complexes which had the capacity to stimulate T-cells. The route taken by the MHC-II/peptide complexes from MiIC's to the cell surface has not yet been described as is the case with the pathway of newly synthesized MHC-II molecules from the Golgi to the MiIC's. Immunoelectron microscopy on B-cells indicates that MHC-II/I-chain complexes are not localized to the same trans-Golgi exit as mannose 6-phosphate receptors. Invariant chain is present in specialized trans-Golgi structures which represent early MiIC stages. The nature of this Golgi to MiIC pathway will be discussed.

1. Harding, C.V., and H.J. Geuze, 1993. Antigen processing and intracellular traffic of antigens and MHC molecules. *Curr. Opinion in Cell Biol.* 5, 596-605.
2. Peters, P.J., J.J. Neeffes, V. Oorschot, H.L. Ploegh, and H.J. Geuze, 1991. Segregation of MHC class-II molecules from MHC class-I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349, 668-675.

P - 6

THE MAJOR HISTOCOMPATIBILITY COMPLEX IN THE LIFE OF A CELL
Jan Klein
Abteilung Immunogenetik, Max-Planck-Institut für Biologie, D-72076 Tübingen

The human major histocompatibility complex (MHC) is spread out over a region of chromosome 6 that is approximately 4000 kb long and contains more than 100 genetic loci. Some of these loci (the MHC proper) are essential for the initiation of the anticipatory immune response in vertebrates; others are part of the effector arm of the immune response, and the remaining loci participate in a variety of housekeeping functions in the life of a cell. The molecules encoded in the MHC proper bind peptides derived from proteins manufactured in the cell (class I molecules) or taken up by the cell (class II molecules) either during their assembly in the endoplasmic reticulum (class I molecules) or in the endosomal compartment (class II molecules). Most of the peptides originate from the cell's own protein (self peptides), but in a cell infected with a parasite, they can be derived from foreign (nonself) proteins. The peptides captured by the specialized peptide-binding region (PBR) of the MHC are displayed on the surfaces of the antigen-presenting cells, which are scanned by T lymphocytes. MHC molecules displaying self peptides are ignored by the T cells, those exhibiting nonself peptides are recognized by the T-cell receptors, and the recognition triggers an immune response specific for the displayed peptide. Among the loci present in the MHC region are also those that may assist in the processing of proteins into peptides taken up by the PBR of the MHC molecules.

The evolutionary history of the MHC poses some interesting questions. How was the complex assembled? Is there a reason for keeping > 100 loci in a tight cluster? Are the loci interdependent in some way? What is the origin of the MHC proper? An attempt will be made to answer these and related questions in the light of our current knowledge of the system.

P - 7

MAP KINASES IN GROWTH FACTOR ACTION
J. Pouysségur, A. Brunet, G. L'Allemand, P. Lenormand and G. Pages
Centre de Biochimie-CNRS, Parc Valrose, 06018, Nice, France

The kinase cascade conserved from fungi to mammals: Raf-1/MAP kinase kinase kinase (MAPKKK)- MAP kinase kinase (MAPKK)- MAP kinase (MAPK)- S6 kinase, plays a major role in the conduction of cell surface receptor-mediated signals to the nucleus. This cascade is activated by the three classes of cell surface receptors (cytokines, tyrosine kinases and G protein coupled receptors). Although the mode of activation at the receptor level differs, it appears that all mitogens clearly activate the two ubiquitously expressed isoforms of MAPK, p42^{mapk} and p44^{mapk}.

We have cloned, epitope-tagged and expressed in fibroblast: the hamster MAPKK and p44 MAPK to analyze their time course of activation, their subcellular localization, their regulatory phosphorylation sites and their role in cell cycle entry. First we show that activation of MAPK is rapid, biphasic and persistent (4 to 6 hrs) and that this kinase cascade is capable to integrate various extracellular signals. Second we demonstrate that activation of MAPKK, that is also rapid (maximum 10-15 min) and persistent (more than 8 hrs) does not show, however, the biphasic activation of MAPK. This result suggest the existence of a very active and specific MAPK phosphate at early phase of activation. In addition we show that an activated form of MAPKK phosphorylates ser222 of MAPK, a key residue switching on and off the kinase. Indeed substitution of ser222 by asp, a negatively charged residue, generates a 'constitutively' active MAPKK. Thirdly we show that both isoforms of MAPK, p42^{mapk} and p44^{mapk}, rapidly translocate to the nucleus in response to mitogens whereas MAPKKK, MAPKK and Raf-1 remain cytoplasmic. This finding, together with the ability of MAPKs to phosphorylate transcription factors, indicate that MAPKs are crucial in relaying external stimuli to the nucleus. Finally, we show that expressing either p44^{mapk} dominant-negative mutations or antisense, suppresses growth factor-induced G0 to S phase transition. We therefore conclude that activation of MAPKs is obligatory for the proliferative response.

P - 8

CYTOSKELETAL PROTEINS AS CELL TYPE-SPECIFIC MARKERS IN CELL BIOLOGY AND PATHOLOGY
Mary Osborn
Max Planck Institute for Biophysical Chemistry, Goettingen, FRG

Of the three major cytoskeletal gene families - actins, tubulins and intermediate filaments - the intermediate filaments seem to be the most useful in distinguishing major cell lineages. Thus antibodies specific for a single intermediate filament polypeptide can be used to determine the types of cells present in primary cultures or to study embryological development. In addition they can be used in the differential diagnosis of different tumor types, and in classifying certain histological and cytologic specimens which do not display perceptible signs of differentiation at the light microscopic level (e.g. Domagala et al., *Cancer* 53, 504, 1989). Antibodies specific for only one of the approximately 30 human keratins can be used to further subdivide carcinomas, or may be of help in subclassifying supposedly homogeneous carcinoma groups such as breast and stomach. Other differential diagnoses can be made by considering coexpression of different IF types and particular emphasis will be placed on coexpression of keratin and vimentin in a subfraction of breast carcinomas. Coexpression of vimentin seems to be a strong indicator of poor prognosis in node-negative NOS breast carcinomas (Domagala et al., *Am. J. Pathol.* 137, 1299, 1990). Other well characterized cytoskeletal proteins, where the expression patterns are restricted to certain cell types, may also have diagnostic value. These include villin which is found in intestinal, renal cell and some stomach carcinomas; nebulin and titin in rhabdomyosarcomas as well as desmoplakins. Limitations and possible pitfalls in using such markers will also be emphasized. Whether nuclear proteins may also have a role to play on cell type specific markers will also be discussed. Emphasis will be placed on the lamins (Röber et al., *J. Cell Sci.* 95, 587, 1990) and on the NuMA/SPN protein (Kallajoki et al., *EMBO J.* 10, 3351, 1991 and *J. Cell Sci.* 104, 139, 1993).

CONSERVATION AND DIVERGENCE OF DNA SEQUENCES
IN PLANT GENOMES**S1-1**

R.B. Flavell

John Innes Centre, Norwich Research Park,
Colney, Norwich, NR4 7UH, UK

The architecture of a genome can be understood much better if the functions and characteristics of its constituent sequences are identified. The sequences of telomeres and coding sequences are highly conserved. Most of the repeated sequences are not highly conserved, in sequence, number or position. Thus genomes consist of sequences evolving at very different rates. The repeated sequences include tandem arrays of units and various kinds of transposable elements. Turnover of the repeated sequences accounts for much of the structural diversity between genomes within species and between related species.

In spite of the structural diversity conferred by the behaviour of families of repeated sequences, the linear order of genes is frequently conserved within large chromosome segments. This discovery, greatly endorsed by the most recent molecular mapping results, provides new understanding on the architecture of genomes and new approaches for the identification and isolation of genes.

The principles defined in this abstract will be illustrated by special reference to plant genomes.

S.cerevisiae: AN ECONOMICAL GENOME?
A-M. Bécam, F. Nasr, Y. Jia, P.P. Slonimski
and C.J. Herbert,
Centre de Génétique Moléculaire du CNRS,
F-91198, Gif-sur-Yvette, FRANCE.

S1-2

The systematic sequencing of the *Saccharomyces cerevisiae* genome has revealed many interesting features which were inaccessible to, or "overlooked" by classical yeast genetics and biochemistry. Since the publication of the sequence of chromosome III many theoretical studies have shown that the nucleotide sequence is non-random in a way that is independent of the coding content. However, at present the functional significance of this is not known. Also, as more sequence data has become available, it has become apparent that the overall coding content of the genome is significantly different from predictions based on classical genetic studies. In particular membrane proteins and duplicated genes are far more common than expected, and large regions of individual chromosomes may also be duplicated. As an example of this we may take the citrate synthase genes.

Two genes encoding citrate synthase (mitochondrial and peroxisomal forms of the enzyme) have been identified and extensively studied in yeast. These genes (*CIT1* and *CIT2*) are included in a large duplication of the centromere regions of chromosomes III and XIV. Between them, these isoforms can account for all the expected cellular functions of citrate synthase. We have identified a third citrate synthase gene (*CIT3*), located close to the centromere of chromosome XVI. Interestingly, *Cit3p* shows comparable levels of similarity when compared to *Cit1p* and *Cit2p* (46 and 47% identity respectively); but this is significantly less than the level of similarity seen between *Cit1p* and *Cit2p* (82% identity).

THE ORGANIZATION OF PLANT CHROMOSOMES

S1-3

J.S. (Pat) Heslop-Harrison

Karyobiology Group, John Innes Centre, Norwich,
NR4 7UJ, UK. FAX: +44 603 56844

Molecular cytogenetic studies allow us to examine and understand chromosome organization and aspects of genome evolution in species with large and small genomes. Repetitive DNA makes up the vast majority of most plant nuclei, and its organization along chromosomes and within interphase nuclei can be studied by DNA:DNA *in situ* hybridization complemented by pulse-field and conventional electrophoresis and Southern hybridization. The localization of the probes on single types or many chromosomes provide indicators of recent and evolutionary rearrangements in the genome, and shows the constraints on dispersion and amplification of the sequences themselves. Comparison of widely different species is enabling definition of common elements of chromosome organization in many species. In collaborative projects, we have been investigating species with some of the smallest known plant genomes - from the genera *Arabidopsis* and *Citrus*, under 200 Mbp long - through beet, with a genome about 1 200 Mbp long, to cereals and pines with genomes up to 25 000 Mbp long. We are building detailed models of large-scale genome structure within individual species, and examining the relationships between repetitive sequences, genes and gene activity. The work is showing how genomes are changing over long-term, evolutionary, and short-term, plant breeding, timescales. Overall, the understanding large scale genomic organization gained using molecular cytogenetic methods will have an impact on strategies for genetic engineering, gene isolation, knowledge of control of gene expression, chromosome recombination and plant breeding.

DNA REPLICATION, THE CELL CYCLE AND
PLANT DEVELOPMENT**S1-4**Dennis Francis, School of Pure and
Applied Biology,
University of Wales, Cardiff, UK.

Two major events of the cell cycle are S-phase and mitosis. In fission yeast, p34 encoded by *cdc2* is central to two important check-points one in late G1 and the other in late G2. Plant homologues to p34 are well-established in a range of unrelated higher plants and the first part of this paper reviews plant genes which function in these check-points.

DNA replication and the duration of S-phase is governed, at least in part, by the amount of nuclear DNA. This is clearly so for species within a ploidy level but less for species of different ploidy. Some time ago¹ we observed a positive relationship between the rate of nuclear DNA replication per single replicon fork and replicon size. A model of DNA replication based on these data will be presented.

The final part of the paper will deal with the onset of mitosis. Recently, we have expressed a mitotic inducer gene, *cdc25* from *S. pombe* in tobacco. The transgenic plants exhibited various developmental abnormalities which indicate that a cell size control maybe central both to the cell cycle and various aspects of plant development.

1. Kidd, A.D., Francis, D. Bennett, M.D.
(1989) *Exp. Cell. Res.* 184, 262-267.

S2-1 STRUCTURE FUNCTIONAL ANALYSIS OF THE
INTERMEDIATE FILAMENT BINDING PROTEIN
PLECTIN
G. Wiche, B. Nikolic, C. Maerker, S. Puchegger, C.G.
Liu, L. Einzenberger

Institute of Biochemistry and Molecular Cell Biology, University of Vienna - Biocenter, 1030 Vienna, Austria

Plectin is an intermediate filament (IF) binding protein of exceptionally large size (> 500kD), that has been proposed to function as a cytoplasmic crosslinking element. Its molecular structure, revealed by electron microscopy and predicted by its sequence, indicates an N-terminal globular domain, a long rodlike central domain and a globular C-terminal domain containing six highly homologous repeat regions. The transient expression of mutant proteins in COS and PtK2 cells showed that the C-terminal domain of plectin, in particular the part containing the last two repeat regions, was indispensable for associations with IFs in living cells. This was confirmed by *in vitro* binding experiments using recombinant plectin mutant proteins expressed in bacteria. Similar analyses indicated that the C-terminal domain harbors also plectin's self-interaction and fodrin binding sites, as well as a specific p34^{cdc2} phosphorylation site. Mitosis-specific phosphorylation of the molecule at this site might play an important role in the dramatic rearrangement of plectin structures observed during the cell cycle, when their filamentous, largely vimentin-associated distribution in interphase changes to a diffuse vimentin-independent cytoplasmic state in mitosis. Structural analysis of the rat genome revealed the existence of at least 11 introns of various lengths, most of which resided upstream of the sequence encoding the rod. Interestingly, the entire rod domain as well as the globular C-terminal domain were encoded by single exons of >3kb and >7kb, respectively. Reverse transcription of mRNA and PCR amplification led to the identification of a differentially spliced transcript variant in several tissues examined. This variant is ~3300 bp shorter than the full length transcript and lacks the entire sequence encoding the rod domain. Currently, a number of genomic cosmid clones cross-hybridizing with plectin cDNA probes are being analyzed whether plectin belongs to a family of distinct genes that are partially related in structure.

S2-2 INVOLVEMENT OF NEUROFILAMENTS IN
AXONAL GROWTH AND IN SELECTIVE
DEGENERATION OF MOTOR NEURONS.

D.W. Cleveland*, M. K. Lee*, J. Marszalek*, and Z.-S. Xu*.
Departments of Biological Chemistry* and Neuroscience*, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 USA.

Neurofilaments are the most abundant cytoskeletal polymers in myelinated axons, particularly in the largest caliber axons such as those of the α motor neurons. Several lines of evidence, including analysis of transgenic mice expressing altered levels of individual neurofilament subunits, have combined to prove that neurofilament accumulation is crucial for the radial growth of axons that normally takes place after stable synapses have formed. Growth in caliber is itself an important event because it is crucial for establishing normal conduction velocity. Despite this important function, there is now direct evidence that aberrant accumulation of neurofilaments can cause human motor neuron disease, including amyotrophic lateral sclerosis (ALS). First, abnormal accumulations of neurofilaments are an early hallmark of the pathogenic process. Second, forced overexpression of neurofilament subunits in transgenic mice models leads to motor neuron dysfunction. Third, expression of modest levels of a point mutant in neurofilament subunit NF-L causes massive, selective degeneration of spinal motor neuron cell bodies and axons. In the few surviving motor neurons, perikaryal and axonal accumulations of neurofilaments are prominent. The neuronal pathology is accompanied by severe denervation-induced atrophy of skeletal muscles. These efforts prove that neurofilament mutations can cause selective motor neuron death and that abnormalities in neurofilaments may be key intermediates in human motor neuron disease.

MICROTUBULE DYNAMICS AND CHROMOSOME MOVEMENT

S2-3 J.R. McIntosh, V.A. Lombillo, E.A. Vaisberg

Dept. Molecular, Cellular, and Developmental Biology, Univ. of Colorado, Boulder, CO 80309, USA

Microtubules (MTs) must polymerize and depolymerize to enable the assembly and action of a mitotic spindle. Moreover, several MT-related motor enzymes have been implicated in mitotic movements. To understand this complexity we are both studying living cells and working with a system that generates chromosome movement *in vitro*. 3-D electron microscopy of mammalian spindles shows changes in MT arrangement that accompany mitosis: elongation, shortening, and sliding. Our work *in vitro* uses a system in which polymerization of brain tubulin is initiated in a flow cell by the basal bodies of detergent-lysed *Tetrahymena*. Mitotic chromosomes from CHO cells bind to the MTs and move as the MTs depolymerize. The movements are ATP-independent and can be even faster than chromosome movement *in vivo*. The forces produced are >1pN, comparable to those produced by spindles. The motions are unaffected by V_0^{3-} , by dynein heavy chain cleavage, or by function blocking antibodies to dynein heavy chain. The motions are stopped by broadly reacting antibodies to kinesin heavy chain and by antibodies specific for the neck region of CENP-E, a large kinesin-like protein localized to kinetochores. HeLa cell kinesin, a plus end directed motor enzyme, will bind to latex spheres and support ATP-independent, minus end-directed motion in association with MT depolymerization. A chimera of the *Drosophila* kinesin head domain and the tail from NCD (a *Drosophila* kinesin-like protein) will move beads at up to 8 μ m/sec, ca. 10X the measured rate of rapid MT shortening. Apparently this chimera can not only transduce the energy from tubulin disassembly into mechanical work, it can catalyze tubulin depolymerization.

S2-4 MICROTUBULE-ASSOCIATED PROTEIN TAU
AND ALZHEIMER'S DISEASE:
STRUCTURE, ASSEMBLY, AND PHOSPHORYLATION.
E. Mandelkow, J. Biernat, G. Drewes, K. Baumann,
O. Schweers, E.-M. Mandelkow
Max-Planck-Unit for Struct. Mol. Biol.,
D-22603 Hamburg, Germany

Microtubules represent the tracks along which intracellular transport of vesicles and organelles occurs in nerve cells, powered by motor proteins such as kinesin or dynein. In the case of axons, the microtubules are stabilized by the "ties" of tau protein, one of the microtubule-associated proteins (MAPs). In Alzheimer's disease, an age-related dementia, one finds tau protein abnormally phosphorylated and aggregated into paired helical filaments, rather than bound to microtubules. To understand the disease process one would like to answer the following questions: Where are the abnormal phosphorylation sites of tau protein? What kinases or phosphatases are involved? What factors are responsible for the aggregation of tau? How do the changes in tau affect its interactions with microtubules or intracellular traffic?

Recent results regarding these questions will be described (for review see TIBS 18:480, 1993). For example, abnormal tau contains two classes of phosphorylation sites. One class of the type Ser-Pro or Thr-Pro and can be phosphorylated by proline directed kinases (MAP kinase, GSK-3, cdk2, cdk5; MAP kinase is the most efficient one of them). The other class contains non-proline directed kinases; they are less efficient but probably more important for microtubule binding (e.g. a kinase affecting Ser262). Both types of phosphorylation sites can be cleared by the phosphatases calcineurin and PP-2A, suggesting that these phosphatases are important for the normal state of tau.

The structure of tau and PHFs can be investigated by several methods, such as spectroscopy, electron microscopy, and X-ray diffraction. Filaments very similar to Alzheimer PHFs can be reassembled *in vitro* from constructs of tau protein which consist essentially of the microtubule-binding domain. This would suggest a direct relationship between microtubule binding and PHF aggregation. Supported by BMFT and DFG.

S3 - 1 THE ROLE OF THE NUCLEAR MEMBRANE
IN THE CONTROL OF DNA REPLICATIONS

Ronald Laskey

Abstract not received

S3 - 2 Nucleocytoplasmic Shuttling of Proteins: a Role in
Transport and Signal Transduction ?

Erich A. Nigg

Swiss Institute for Experimental Cancer Research (ISREC),
155, Chemin des Boveresses, CH-1066 Epalinges, Switzerland.

The existence of proteins that migrate constantly between nucleus and cytoplasm (shuttling proteins) was first revealed by transplantation experiments in *Ameoba proteus* (e.g. Goldstein 1958). However, the molecular identification of such proteins in vertebrates had to await the development of sensitive methods (Borer et al. 1989). Recently, shuttling proteins have attracted considerable interest because they might conceivably function as carriers in nucleocytoplasmic transport processes, and they might contribute to coordinating nuclear and cytoplasmic activities (for reviews see Goldfarb 1991, Nigg 1992, Laskey and Dingwall 1993). Our most recent studies revealed that nucleocytoplasmic shuttling is a general phenomenon not restricted to proteins involved in nucleocytoplasmic transport, and that the steady-state distribution of a shuttling protein is not governed exclusively by the relative rates of nuclear import and export, but also by its relative affinities for cytoplasmic and nuclear binding partners (Schmidt-Zachmann et al. 1993). Thus, protein shuttling between nucleus and cytoplasm may constitute a major dynamic element in the regulation of both nuclear and cytoplasmic activities.

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S3 - 3 ASSEMBLY AND NUCLEO-CYTOPLASMIC TRANSPORT
OF UsnRNPs

R. Lührmann, Institut für Molekularbiologie und Tumorforschung
der Philipps-Universität Marburg, Emil-Mannkopff-Straße 2,
D-35037 Marburg, Germany

Biogenesis of spliceosomal UsnRNPs involves migration of newly transcribed m⁷G capped UsnRNAs to the cytoplasm, assembly with the core snRNP proteins (denoted B/B', D1, D2, D3, E, F, and G), trimethylation to the m₃GpppN cap structure and finally transport back to the nucleus. The nuclear location signal (NLS) of U1 snRNP, as investigated by microinjection in *Xenopus laevis* oocytes, is bipartite, with the m₃G cap structure being one essential element. The second part of the NLS resides on the snRNP core RNP structure.

Surprisingly, the signal requirement for nuclear transport of U1snRNP was shown to differ between oocytes and somatic cells in that the m₃G-cap structure is no longer an essential signalling component. However, as shown by analysing the transport kinetics of m₃G- and ApppG-capped U1snRNPs the m₃G-cap structure accelerates the transport significantly. As a prerequisite for a more detailed investigation of the mechanism of nuclear snRNP import and for the characterization of the transport factors involved, we have established an *in vitro* snRNP nuclear import system using digitonin permeabilized somatic cells supplemented with cytosolic extracts. Nuclear import of snRNPs, in permeabilized NRK cells supplemented with somatic cell cytosol, requires the same NLS structure as those identified in micro-injected mammalian cells. Interestingly, when the *in vitro* system was provided with cytosol from *Xenopus* oocytes instead of somatic cells, U1 and U2 snRNP nuclear import was m₃G-cap dependent. These results indicate that soluble cytosolic factors mediate the differential m₃G-cap dependence of U1 and U2 snRNP nuclear import in somatic cells and oocytes. We are currently investigating the contribution of individual core snRNP proteins to the nuclear transport.

As a prerequisite to investigate the assembly pathway of the core RNP domain, we have cloned the cDNAs for the human Sm proteins D2, D3, F and G. We have studied the interactions between these proteins and proteins E, F and B/B' in the absence of RNA by co-immune precipitation analysis of protein-protein hetero-oligomers. Proteins translated *in vitro* were used. Our results suggest that a protein-heterooligomer consisting of proteins E, F, G, D1, D2 binds first to the snRNA, after which addition of a B/B'-D3 complex takes place.

Finally, the structural requirements for the trimethylation of the snRNA's cap structure *in vitro* and the initial characterization of the UsnRNA-(guanosine-N2)-methyl-transferase will be reported.

S3 - 4 TRANSCRIPTION AND NUCLEAR ARCHITECTURE

D.G. Wansink^a, O.C.M. Sibon^b, E.M.M. Manders^c, B. van Steensel^a, M. Grande^a, B. Humber^b, J.A. Aten^a, R. van Driel^a, L. de Jong^a

^a E.C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, 1018TV Amsterdam, The Netherlands; ^b Dept. of Molec. Cell Biol. University of Utrecht, Utrecht; ^c Dept. of Radiotherapy, University of Amsterdam, Amsterdam

We have recently developed a method to fluorescently label nascent pre-mRNA in interphase nuclei (Wansink et al. (1993) J. Cell Biol. 122, 283-293). Double labelling procedures, using antibodies that recognize (i) nascent DNA, (ii) an essential splicing factor and (iii) transcription factors, like the glucocorticoid receptor, give important information about the architecture of the interphase nucleus. Among others, we find that, despite the fact that splicing at least partially is a cotranscriptional process, the sites of transcription do not coincide with nuclear domains that contain a high concentration of splicing machinery. Also, no extensive colocalization was observed between transcription and domains of early or late DNA replication. Recently we have extended the pre-mRNA labelling procedure to the electron microscopic level. Results indicate that transcription by RNA polymerase II is localized at the surface of what seem to be local areas of more compacted chromatin.

Many nuclear functions and domains are associated with the nuclear matrix. This structure remains after removal of most of the chromatin and soluble proteins from the nucleus. Evidently, the nuclear matrix plays an important role in structuring the nucleoplasm.

Our results, combined with the data from others, begin to give insight in the functional organization of the cell nucleus.

S4-1 WATER CHANNELS IN CELL MEMBRANES

Gh. Benga

Dep. Cell Mol. Biol., Univ. Med. Pharm., Cluj-Napoca, Romania

A systematic programme of comparative nuclear magnetic resonance measurements of the membrane permeability for water diffusion (P_d) and of the activation energy ($E_{a,d}$) of this process in red blood cells (RBCs) from various wild, laboratory and domestic animal species was accomplished. The RBCs from human, cow, sheep and kangaroos had P_d values around 5×10^{-3} cm/sec at 25°C , 7×10^{-3} cm/sec at 37°C and $E_{a,d}$ values around 25 kJ/mol. For the RBCs from other ten marsupial species or from mouse, rat and rabbit, the P_d values were more than twice as high as for human RBCs. For most species of RBCs a high value of P_d was associated with a low value of $E_{a,d}$ (ranging from 15 to 21 kJ/mol) pointing to specialized channels for water diffusion incorporated in membrane proteins.

Recently a channel-forming integral protein of 28 kD (CHIP 28) was identified as a major water channel protein in the RBC membrane. A procedure for quantitating the purified CHIP 28 by densitometry of silver stained polyacrylamide gel electrophoregrams was developed. The analysis of a purified fraction of CHIP 28 showed that the 28 kD component represents approximately two thirds of the sample with remainder comprising the glycosylated high molecular weight component. A correlation between the content in CHIP 28 and the relative water permeability among RBCs from different vertebrate species was attempted.

S4-3 ABC TRANSPORTERS - FROM MICROORGANISMS TO MAN

F. Higgins

Abstract not received

S4-2

THE MOLECULAR BIOLOGY OF TRANSPORT PROTEINS FOR SUGARS AND ANTIBIOTICS

Peter J.F. Henderson

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

Many of the transport proteins found in the membranes of mammalian cells and their organelles have homologous equivalents in microorganisms. For example, the human glucose transporters (GLUT1-7) are members of a superfamily, which includes transporters for galactose (GalP), arabinose (AraE) and xylose (XylE) found in *Escherichia coli*, and some mammalian neurotransmitter transporters are homologous to microbial antibiotic transporters. Statistical comparisons of the amino acid sequences of 58 transport proteins suggest the existence of at least four families related to GalP/AraE/XylE: one for sugars; one for carboxylates; one for antibiotics or antiseptics such as methenomycin, actinorhodin and ethidium; and one for quinolones, tetracyclines and chloramphenicol. The hydrophobic profiles of these proteins usually predict that they contain 12 membrane-spanning α -helices. This 12-helix theme applies also to other transport proteins of apparently unrelated sequences, including those for lactose (LacY), fucose (FucP), glucuronides (GusB), proline (PutA) and proline/betaine (ProP) in *E. coli*, and for glucose-Na (SGLT1) or lactate (MCT1) in mammals, which are similar to PutA and ProP. We have amplified expression of each of the *E. coli* GalP, AraE, XylE, GusB, FucP and ProP transporters, and of the *Bacillus megaterium* Bmr antibiotic transporter, so they constitute 8-66 % of the inner membrane protein. These levels permit molecular analysis of their structure-function relationships using various techniques. The results are interpreted in terms of a unifying structural model of microbial and mammalian transport proteins. This research is supported by SERC, SmithKline Beecham plc and the Wellcome Trust

S4-4 THE H⁺-ATPASE FROM YEAST PLASMA MEMBRANE: RECENT PROGRESSES.

A. Goffeau, M. Boutry, I. Degand, A. de Kerckhove, F. Catty, E. Estrada, C. Navarre, P. Supply and A. Wach.

FYSA, Université de Louvain, I348 Louvain-la-Neuve, B.

Yeasts contain a H⁺-pumping ATPase. The biochemical characterisation of this enzyme carried out during the last 15 years has resolved the major steps of the catalytic cycle for ATP hydrolysis. We will report on three major problems largely unsolved today.

1. Role of Histidine residues in H⁺ transport

The best six conserved His residues in all H⁺-ATPases sequenced so far have been mutated by site directed mutagenesis. Only His 701 was found to be essential for growth and no tested substitution was allowed. The H285E substitution was not allowed whereas H285Q and H285R were viable but their ATPase activity was modified in several aspects. The other tested substitutions had no effect on yeast growth.

2. Metabolic regulation of H⁺-ATPase activity

Two small proteolipids PMP1 and PMP2 located in the plasma membrane were found to be physically associated to the H⁺-ATPase protein. The double deletion of the PMA1 and PMA2 genes resulted in a decreased V_{max} of the H⁺-ATPase.

The H⁺-ATPase protein was found to be phosphorylated by a casein kinase (type I) located in the plasma membrane. This kinase activity which is encoded by the YCK 1 and/or YCK 2 genes is lowered by glucose activation.

3. Subcellular traffic of H⁺-ATPase

Upon overexpression of the H⁺-ATPase genes PMA1 or PMA2 and of several pma1 mutants, the H⁺-ATPase molecules accumulate within proliferating endoplasmic reticulum (PER) structures.

S5 - 1

MOLECULAR BASIS OF BASEMENT MEMBRANE FUNCTIONS

R. Timpl

Max-Planck-Institut für Biochemie,
Martinsried, Germany

Basement membranes are ubiquitous extracellular protein matrices which control several cellular functions through the binding to cellular receptors. Major constituents of these matrices are various isoforms of laminin and collagen IV, proteoglycans including perlecan, nidogen and fibulins. Laminin and collagen IV form large irregular networks to which the other proteins are bound. Nidogen seems to play a crucial role in the supramolecular organization and mediates the connection of the networks and other heterotypic interactions. Several of the proteins or of their binding domains are now available in recombinant form and were used for a precise mapping of binding sites. This showed that nidogen binds to a single EGF-like repeat of laminin $\gamma 1$ chain. Studies with synthetic peptides and mutants allowed to identify several amino acid residues which are essential for these interactions. Laminins, collagen IV, perlecan and fibulins-1 and -2 function also as major cell adhesion substrates. The binding sites involved are distinct from those used in matrix interactions and recognized by different sets of integrin receptors.

S5 - 3 THE ROLE OF INTEGRINS IN REGULATING KERATINOCYTE PROLIFERATION AND TERMINAL DIFFERENTIATIONF.M. Watt

Keratinocyte Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London. WC2A 3PX, England.

Extracellular matrix receptors of the integrin family are expressed by keratinocytes in the basal layer of the epidermis and are absent in the suprabasal layers where keratinocytes undergo terminal differentiation. The receptors not only anchor basal keratinocytes to the underlying basement membrane, but also play a role in regulating the onset of terminal differentiation and in movement of cells from the basal to the first suprabasal layer. Integrins are also believed to mediate lateral cell migration during wound healing and to participate in intercellular adhesion. Furthermore, different subpopulations of basal keratinocytes (namely, stem cells, transit amplifying cells and cells committed to undergo terminal differentiation) can be distinguished on the basis of differences in the number and state of activation of integrins.

S5 - 2 LAMININ-1 AS A REGULATOR OF EPITHELIAL CELL DEVELOPMENTP. EKBLOM

Department of Animal Physiology, Uppsala University, Box 596, S-751 24 Uppsala, Sweden

Branching epithelial morphogenesis in the embryo is dependent on interactions between the surrounding mesenchyme and the epithelial cells. Our studies suggest that laminin-1, a basement membrane glycoprotein is involved in epithelial-mesenchymal interactions. Laminin-1 (formerly called EHS-laminin) is a glycoprotein with three different chains, $\alpha 1$, $\beta 1$ and $\gamma 1$ (previously called A, B1 and B2). The laminin $\alpha 1$ chain was shown by antibody staining and in situ hybridization to be produced mainly by embryonic epithelial cells. Domain-specific antibodies were used in organ culture of mouse embryonic tissues to study the function of laminin-1. Previous studies have shown that antibodies against domains E3 and E8 affect epithelial cell development in organ culture of kidney. Our recent results from the embryonic kidney and salivary gland suggest that the domains have distinct functions. The E3 domain appears to be important for initiation of basement membrane formation, whereas the E8 domain is involved in binding of laminin to the cell surface. Several authors have shown that the E8 domain binds to integrin $\alpha 6\beta 1$ and our data suggest that this interaction is important for epithelial cell development. Laminin-1 is known to also bind to nidogen, another basement membrane component. We have found that antibodies against the nidogen-binding site of laminin-1 perturb development in organ culture. Interestingly, nidogen was found to be produced by mesenchymal rather than epithelial cells. It is therefore suggested that epithelial-mesenchymal interactions in part are mediated by the interaction between nidogen derived from mesenchyme and laminin-1 derived from epithelium.

S5 - 4 GROWTH FACTORS AND CELL ADHESION MOLECULES AS MORPHOREGULATORSJ.P. Thiery, A. Beauvais, B. Boyer, S. Dufour, C.

Erickson, J. Jouanneau, and A. M. Vallés

CNRS and Ecole Normale Supérieure, 46 rue d'Ulm - 75230 PARIS Cedex 05 FRANCE

The role of cell-substrate adhesion during neural crest cell migration was approached by injecting S180 sarcoma cells in the chick embryo at the sites of neural crest departure. Non-transfected S180 cells mimicked the behavior of normal crest cells, migrating along the ventral and lateral pathways followed by crest cells. Transfected S180 cells expressing high levels of $\alpha 5\beta 1$ integrin, which is known to interact specifically with the central binding domain of fibronectin (FN), spread and migrated faster on FN than control cells in vitro. In vivo, the transfected cells migrated precociously along the subectodermal pathway, emphasizing the role of $\alpha 5\beta 1$ in the pathfinding mechanisms.

The inductive mechanisms implicated in the modifications of the adhesive status of the embryonic cells allowing their dispersion, migration and morphogenetic transformations was also studied by using an epithelial tumor cell line (NBT-II) which can convert into motile fibroblastic-like cells upon exposure to acidic FGF or collagens. We will describe early events in the transduction pathways activated by aFGF signalling through a tyrosine kinase surface receptor and by collagens through $\alpha 2\beta 1$ integrin.

CONTROLLING CELL FORM IN FISSION
S6 - 1 YEAST

P. Nurse, F. Chang, J. Mata, V. Snell and F. Verde, Cell Cycle Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX

The fission yeast cell grows as a cylinder, which begins its cell cycle by growing from one tip, switches on growth at the other tip some way through the cell cycle and divides by medial fission. Morphological mutants have been isolated in order to identify the genes involved in the generation of cell form and to establish what rules of cellular morphogenesis may apply to the growing fission cell. These mutants fall into several categories. Firstly, mutants which are spherical and unable to generate polarised growth. They define 12 orb genes one of which encodes casein kinase II. Secondly, T-shaped mutants which activate supernumery growing points. These define 3 tea genes, mutants in one of which result in growth at only one tip at a time. Thirdly, bent or banana shaped mutants which do not grow as regular cylinders. Fourthly, mutants which are unable to determine their middles during cell division and therefore divide asymmetrically. Study of these mutants is revealing about the rules underlying morphogenesis in fission yeast.

S6 - 2 APICAL CELL JUNCTIONS AND SIGNALLING PATHWAYS IN DROSOPHILA EPITHELIUM
Peter J. Bryant, Developmental Biology Center, University of California, Irvine, CA 92717, USA

Genetic studies in *Drosophila* are revealing that apical junctions between epithelial cells play dynamic roles in cell communication as well as structural roles. In the *wingless/armadillo* signalling pathway, the *Delta/Notch* pathway, and the *boss/sev* pathway, proteins that mediate the cell interaction or initiate signal transduction are localized at adherens junctions. Furthermore, each of these cell interactions appears to involve the transfer of the signalling molecule from the signalling cell into the receiving cell. Septate junctions, just basal to adherens junctions, may also play a role in cell interactions as indicated by the presence in septate junctions of the protein product (Dlg) of the *dlg* tumor suppressor gene. Dlg is required for the formation of septate junctions, and for the localization at septate junctions of the transmembrane molecule fasciclin III. One of the mammalian homologs of Dlg is localized in synaptic junctions, two are localized in tight junctions, and one is a component of the erythrocyte membrane cytoskeleton. All of these proteins have a domain with homology to guanylate kinase, but some residues critical for interaction with substrates are missing, suggesting that the catalytic role of this domain has changed during evolution. Homozygous loss of the tumor suppressor gene *warts* in mitotic recombination clones leads to clone rounding, splitting and overgrowth. It also causes expansion of the apical cell domain, and allows cuticle to be deposited between epithelial cells as well as over their apical surfaces. The predicted product is homologous to the human myotonic dystrophy kinase, and to the *Neurospora* cot-1 protein. Mutations in the cot-1 gene also change cell shape by increasing the number of hyphal branch points. Thus genetic analysis has identified proteins with important structural roles in cell polarity, and has led to the identification of signalling pathways that are mediated by proteins localized at junctions.

Trafficking of immunoglobulin receptors in epithelial cells:
signals and cellular factors

S6 - 3 Walter Hunziker, C. Fumey, S. Höning, and C. Kamel. Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland.

To generate and maintain a polarized plasma membrane protein distribution, epithelial cells take advantage of both the biosynthetic and endocytic pathways. Signals in the cytoplasmic domain of newly synthesized membrane proteins specify vectorial sorting from the trans-Golgi network to the basolateral surface of MDCK cells. While some basolateral sorting determinants are co-linear with the coated pit localization signal, others are distinct. In both cases, tyrosine-dependent and -independent determinants exist. The tyrosine-independent endocytosis and basolateral sorting signal in the cytoplasmic domain of the macrophage IgG FcRII-B2 was now found to be based on a novel di-leucine motif. This finding underscores the close relationship between basolateral sorting and coated pit localization signals and suggests that tyrosine and di-leucine based motifs can mediate endocytosis, basolateral sorting and transport from the TGN to endosomes.

In the endocytic route, proteins are sorted in endosomes for recycling, lysosomal transport, or transcytosis. Brefeldin A has been shown to affect the morphological integrity of endosomes and specifically inhibit basolateral to apical transcytosis via the polymeric immunoglobulin receptor (pIgR). We now find that the drug impairs the segregation in endosomes of the recycling transferrin receptor from the transcytotic pIgR. Furthermore, we provide evidence for an important role of rab17 in regulating transcytosis via the pIgR.

Newborn rodents acquire passive immunity by absorbing maternal milk IgGs across the gut. This uptake is mediated by an IgG Fc receptor (FcRn) related to MHC class I. To obtain a marker protein to study apical to basolateral transcytosis, we used PCR to isolate the FcRn cDNA. This led to the identification of a novel FcRn isoform generated by alternative splicing. Although the spliced FcRn lacks the extracytoplasmic alpha-2 domain, it was able to bind and internalize IgG in a pH-dependent fashion. This finding has implications with respect to the structure and function of MHC class I molecules and suggests that multiple FcRn isoforms may be involved in IgG transport across the neonatal intestine.

(Supported by the Swiss National Science Foundation)

FROM EPITHELIAL TO NEURONAL POLARITY

S6 - 4 K. Simons, C. Dotti, K. Fiedler, M. Murata and J. Peränen. European Molecular Biological Laboratory, Meyerhofstraße 1, Heidelberg, Germany 69012

We have shown that the sorting of newly synthesized proteins to the polarized cell surface in epithelial cells and neurons exhibits remarkably similarities. The basolateral plasma membrane domain seems equivalent to the somatodendritic cell surface while the apical plasma membrane corresponds to the axolemma. We are now dissecting the mechanisms involved in sorting and targeting. Several lines of evidence suggest that the sorting into the apical pathways in MDCK cells is based on specific recognition events. Not only do apical proteins have to be recognized by specific sorting proteins but glycosphingolipids and glycosylphosphatidyl inositol (GPI)-anchored proteins are also included into the membrane patch that forms the transport vesicle. The sorting process was postulated to proceed by protein-protein and protein-lipid interactions. We have proposed that glycolipids associate with each other to form rafts and that these constitute sorting platforms onto which machinery proteins and cargo is incorporated. One prediction of the glycolipid-based sorting hypothesis is that there should be specific interactions between glycolipids and some apically directed proteins or vesiculation machinery. This prediction was confirmed when we investigated the detergent solubility properties of influenza HA and the apical vesicle proteins. Indeed, influenza HA together with a subset of these vesicle proteins and glycolipids was found not to be solubilised by the detergent CHAPS while the basolateral marker protein, VSV-G was. Based on this remarkably specific insolubility we have isolated three membrane proteins VIP21 - caveolin, VIP36 and VIP17 from the carrier vesicle. The properties of these three proteins will be described and their function in epithelial and neuronal protein sorting will be discussed.

S7 - 1 CONTROLLING G1 PROGRESSION IN FISSION YEAST

P. Nurse^a, J. Correra^a, S. Moreno^b and B. Stern^a
^a Cell Cycle Laboratory, Imperial Cancer

Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX;

^b Departamento de Microbiología y Genética, Universidad de Salamanca, Campus Miguel de Unamuno, Avda Campo Charro Sin, 37007 Salamanca, Spain

The *rum1* gene in fission yeast encodes a 25kD protein with a key role in controlling G1 progression. Firstly, it acts as the major rate limiting step which determines the length of G1. In the absence of *rum1* cells are advanced prematurely through G1 into start, the point of commitment in the cell cycle where the programme of events leading to S-phase and mitosis is initiated. Secondly, it acts in the checkpoint control which restrains mitosis during the pre-start G1 interval. Cells lacking *rum1*, prematurely activate the p34cdc2/cyclin B mitotic kinase when arrested pre-start. *rum1* encodes a potent inhibitor of the kinase which when absent allows the kinase to be inappropriately activated. Thirdly, high expression of *rum1* reprogrammes a G2 cell to re-enter the pre-start G1 interval. As a consequence the cell becomes locked into an endo-reduplication state leading to a massive increase in DNA content.

S7 - 2 UNSTABLE PROTEINS AS MITOTIC REGULATORS IN *DROSOPHILA*: CYCLINS AND NOVEL CANDIDATES

C.F. Lehner, H. Jacobs, J. Knoblich,
S. Sigrist and R. Stratmann

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37/39,
72076 Tübingen, FRG

Gene products required for cell cycle progression in the early embryo are derived from stockpiles provided by the mother during oogenesis. Phenotypic manifestations in progeny homozygous for mutations in the corresponding genes start only after the exhaustion of this maternal provision. While in case of stable proteins (e.g. cdc2 kinase) maternal provisions are usually sufficient for all of embryogenesis, this is not the case with unstable proteins (e.g. cyclin A). Null mutations in genes encoding unstable regulators are expected to cause defects early in development. Therefore, we have concentrated on genes with mutant phenotypes already during embryonic cell cycle progression. Two of these genes, *three rows* and *pimples*, encode novel proteins specifically required for sister chromatid separation in anaphase. In addition, we will present evidence indicating that cyclin A degradation is required for anaphase.

S7 - 3 CDK-ACTIVATING KINASE (CAK) : IDENTIFICATION OF THE TWO SUBUNITS COMPLEX AND REGULATION OF ITS ACTIVITY

D. Fesquet^a, J.M. Blanchard^b, J.C. Cavadore^a, J.M. Darbona^a, A. Devault^a, J.C. Labbé^a, A.M. Martinez^a and M. Doreé^a

^aCNRS UPR 9008 and INSERM U 249, BP 5051, 34033 Montpellier Cedex, France ; ^bCNRS UPR 9942 BP 5051, 34033 Montpellier Cedex, France

It has become apparent in the past few years that a central and rate-limiting control function in most transitions of the cell cycle is performed by protein kinase complexes between cdc2, or one of its relatives in higher eukaryotes, and a member of the divergent cyclin family.

Phosphorylation of Thr 161 of human cdc2 and its homologues in the family of others cyclin-dependent kinases (cdks) is required for catalytic activity of cdks. The kinase responsible for phosphorylation and full activation of cdks has been purified from starfish and *Xenopus* oocytes and shown to contain a cdc2-related protein encoded by the MO15 gene as a catalytic subunit. The regulatory subunit has been cloned and sequenced, and the affects of its over-expression investigated. Phosphorylation of at least one residue (Thr 176 in *Xenopus*) by an upstream kinase is required for the MO15 gene product to express its CAK activity. The MO15 gene product contains a canonical nuclear localization conserved from starfish to human, and its translocation into the cell nucleus is required for CAK activity to be generated. The nuclear localization of CAK may imply that cdks activation primarily occurs in the nucleus of oocytes and somatic cells.

CELL CYCLE CONTROL IN BUDDING YEAST

S7 - 4 Kim Nasmyth

Abstract not received

S8-1 THE CONTROL OF CELL FATE AND CELL MOVEMENT BY EXTRACELLULAR SIGNALLING MOLECULES DURING THE MORPHOGENESIS OF DICTYOSTELIUM
J.G. Williams, N.A. Hopper, A. Early, T. Abe,
and M. Nelson

Imperial Cancer Research Fund, Clare Hall Laboratories,
 South Mimms, Herts., EN6 3LD U.K.

During formation of the Dictyostelium slug extracellular cAMP signals direct the differentiation of prespore cells and DIF, a chlorinated hexaphenone, induces the differentiation of prestalk cells. We present a model for cell movement and cell type regulation that begins to explain how the slug is formed, how it maintains a constant ratio of the constituent cell types and how it becomes transformed into a mature fruiting body. We also describe some of the components of the intracellular signalling pathways that mediate these processes.

S8-2 ABERRANT MORPHOGENESIS IN DICTYOSTELIUM MUTANTS DEFECTIVE IN ACTIN BINDING PROTEINS
A. A. Noegel, M. Haugwitz, B. Köppel, J. Karakesisoglou¹,
U. Gottwald and M. Schleicher

Max-Planck-Institut für Biochemie, 82152 Martinsried, F.R.G., ¹Ludwig-Maximilians-Universität, Institut für Zellbiologie, 80336 München, F.R.G.

Actin filaments are involved in many cellular processes, in cell shape formation and in cell motility. In order to perform these functions, a tight control of filament polymerization and depolymerization is required. This regulatory function is performed by actin binding proteins. Several actin binding proteins have been identified in *Dictyostelium discoideum* whose in vitro activities have been characterized and their domain structures have been elucidated. Many of them are members of families which are highly conserved during evolution. To investigate the role of specific actin binding proteins for cell shape, motility and development, mutants have been generated using homologous recombination to inactivate the corresponding genes. The mutants lacked a) the two profilin isoforms and b) α -actinin and gelation factor (ABP120). These proteins interact with the actin network in different ways. Profilins bind to G-actin and influence the state of actin assembly, α -actinin and gelation factor stabilize an existing network thereby influencing the cytoplasmic viscosity. Inactivation of the two profilin isoforms led to alterations at the single cell level and during development. Mutants could not be grown in shaking cultures and grew very slowly on a lawn of bacteria. Development was delayed with regard to the onset of chemotactically induced aggregation, prestalk and prespore cells were formed, but further development was blocked. In synergy experiments approx. 30 % of wild type cells were sufficient to overcome the developmental defect. Mutants lacking the two crosslinkers showed also alteration at the single cell level and during development. A clear defect in cellular motility was noted with a reduction in speed and abnormal pseudopod formation. The block in development occurred already at the aggregation stage and differentiation into prespore and prestalk cells did not take place. The data suggest that the multicellular stage is severely affected by alterations in the cytoskeleton and that an intact cytoskeleton is necessary for completion of the developmental cycle.

S8-3 CELLULAR AND GENETIC EVENTS DURING DROSOPHILA GASTRULATION

M. Leptin, M.C. Stella, J. Casal, C. Esguerra, U. Irion
 Max Planck Institut für Entwicklungsbiologie,
 Spemannstr. 35, 72076 Tübingen, Germany

The first event of *Drosophila* gastrulation, the formation of the ventral furrow, is brought about by cell shape changes within the epithelium on the ventral side of the embryo. Ventral cells constrict their apical ends as their nuclei move towards the basal end. At the same time, myosin is relocated from the basal to the apical end suggesting a role for myosin in the process of apical constriction. In *twist* and *snail* mutants, in which apical constriction fails to occur, incomplete or no myosin relocation is observed. Since the products of the *twist* and *snail* genes are transcription factors, they cannot directly affect myosin behaviour, but must do so via the regulation of other genes. We have made a subtractive library enriched for cDNAs of genes expressed under the control of *twist* and *snail*. We have isolated a large number of clones which represent at least 14 new genes expressed on the ventral side of the embryo and in mesodermal derivatives. Sequencing and genetic analysis are being used to study their roles in gastrulation and mesoderm differentiation.

S8-4 SEGMENTATION AND CELL ARCHITECTURE IN DROSOPHILA EMBRYOS

H. Francis-Lang, I. Davis and D. Ish-Horowicz

Developmental Genetics Laboratory, Imperial Cancer Research Fund Developmental Biology Unit, Zoology Dept., Oxford University, Oxford, UK.

Antero-posterior patterning in *Drosophila* is a process of transcriptional regulation within a single syncytial cell, the multi-nucleate blastoderm embryo. Spatial distinctions within the cell arise from a regulatory cascade of segmentation genes that are expressed in successively more precise domains. Metameric (reiterated) pattern depends on the pair-rule genes, the first to be expressed in stripes. Pair-rule transcripts show a secondary form of intracellular localisation, accumulating exclusively apically of the layer of blastoderm nuclei. This localisation is specific and is directed by 3' signals in the RNA, (Davis and Ish-Horowicz, Cell 67, 927: 1991).

We have tested whether pair-rule transcripts are localised apically by transport within the cytoplasm or by vectorial nuclear export. We examined pair-rule transcripts in mutant embryos with disrupted cytoarchitecture. Embryos lacking chromosome arm 3L, are defective in maintaining nuclei at the cell periphery; some cortical nuclei become displaced towards the egg interior, although retaining transcriptional activity and apico-basal orientation. Consistent with the vectorial nuclear export model, pair-rule transcripts accumulate apically of internalised nuclei, i.e. basal of surface nuclei. This localisation does not correlate with associated cytoarchitecture. In particular, internalised nuclei lack the apical "basket" of microtubules present over wild-type nuclei. Sites of pair-rule transcript localisation correlate with nuclear orientation, although localisation is independent of genes' positions within the nucleus (Davis *et al.*, CSHSQB, 58, in press: 1993). We discuss these and other results in terms of mechanisms of transcript export and the role of transcript localisation in patterning the *Drosophila* embryo.

S9-1 EARLY EVENTS IN ANTIGEN PRESENTATION
J. J. Neefjes

Abstract not received

S9-2 FACTORS DETERMINING THE PRESENTATION OF
PEPTIDE EPITOPES WITH MHC CLASS I

K. Eichmann, Gabi Niedermann, Maria Lucchiari,
Carola Leipner and Bernhard Maier

Max-Planck-Institut für Immunobiologie, Postfach
1169, D-79011 Freiburg, FRG

Cytotoxic T lymphocytes recognize peptides derived from proteins which are endogenously synthesized and presented with MHC class I molecules. Activation of cytotoxic activity following recognition by CTL precursors is considered to be a main mechanism in immunity against viruses and against tumor cells. Presentation of peptides with MHC class I requires the proteolytic degradation of proteins within the cytoplasm, presumably by proteasomes; this is followed by peptide transport into the endoplasmic reticulum with the help of the TAP transporters; transported peptides are then complexed with MHC class I molecules within the endoplasmic reticulum and these complexes are subsequently deposited on the outer cell membrane. Major rate limiting events in this process are the proteolytic cleavage of proteins, the peptide transport into the ER as well as the complexation with MHC molecules. We will describe studies on the role of amino acid sequences flanking potential MHC class I epitopes which suggest that these sequences may present limiting factors in the generation of the epitope by proteolytic cleavage. This rate limiting role of flanking sequences may be of importance in the determination of the degree of immunodominance of an epitope. Our studies show that a subdominant protein epitope can be converted into an immunodominant one when fitted with the flanking amino acid sequences of an immunodominant epitope. Conversely, an immunodominant epitope can be converted into a subdominant epitope when fitted with the flanking sequences of a subdominant epitope. These studies may shed a new light on the long-standing problem of epitope hierarchy in cell mediated immunity and may be useful for the design of peptide vaccines.

S9-3 THE INTERACTION BETWEEN PEPTIDE AND MHC
CLASS I
Soren Buus

Abstract not received

S9-4 EFFICIENT MHC CLASS II-RESTRICTED
PRESENTATION OF MEASLES VIRUS TO
ANTIGEN SPECIFIC T CELLS RELIES ON ITS
TARGETING TO ITS CELLULAR RECEPTOR
HUMAN CD46 AND INVOLVES AN
ENDOSOMAL PATHWAY.

D. Gerlier^a, G. Varior-Krishnan^b, M.-C. Trescol-Biémont^b, D. Naniche^b, I. Fugier-Vivier^b & C. Rabourdin-Combe^b.

^aImmunité & Infections Virales, I.V.M.C., CNRS-UCBL UMR 30,
Faculté Alexis Carrel, 69372 Lyon Cedex 08, & ^bImmunobiologie
Moléculaire, CNRS-ENS UMR 49 69364 Lyon Cedex 07, France.

The induction of specific immunity involves the stimulation of B and T lymphocytes. In contrast to the B-cell antigen-specific receptor (Immunoglobulin), that of T cells (TcR) recognizes an antigen not directly but indirectly as a bimolecular complex expressed at the surface of an antigen presenting cells (APC). This complex associates a peptide derived from the intra-cellular degradation of the antigen and a Major Histocompatibility Complex (MHC) molecule. CD4 T lymphocytes recognize peptide-MHC class II complexes expressed by professional APC. The role of the measles virus (MV) receptor, human CD46, in the uptake of MV antigens and their presentation by MHC class II molecules was investigated. Expression of CD46 in murine B cells resulted in cells highly efficient in capturing UV-inactivated MV particles and presenting both envelope hemagglutinin H and nucleoprotein N. Although MV fuse with the plasma membrane of its target cells, presentation of both MV-H and -N to T cells were sensitive to inhibition by chloroquine. However, whereas 50 µM of chloroquine was required to abolish presentation of MV-H, purified H or soluble N, a two-fold lower concentration was required to inhibit that of MV-N. This shows that CD46-mediated captured MV particles should have been endocytosed then processed in an endosome/lysosome compartment. Since CD46 is expressed in almost every human tissue, including professional APC, such a targeting should be important in the human CD4+ T cell-mediated primary immune response against MV.

S10-1 GENETIC CONSTITUTION OF TRANSGENIC PLANTS
Y. Y. Gleba

Abstract not received

S10-2 MOLECULAR APPROACHES TO GENETIC MANIPULATION
OF PHOTOOXIDATIVE PARTITIONING USING
TRANSGENIC PLANTS

L. Willmitzer

Abstract not received

S10-3 ENGINEERING HERBICIDE RESISTANCE: THE AHAS
PARADIGM
R. Chaleff
American Cyanamid, Agricultural Research
Division, P.O. Box 400, Princeton, New
Jersey, USA

Crop varieties with enhanced tolerance for several classes of herbicides have been produced by three different methodologies: selection in cell culture, mutation breeding, and introduction of foreign genes by genetic transformation. Examples of resistance to glyphosate, phosphinotricin, sethoxydim, picloram, and sulfonylureas are presented. The use of these methodologies to introduce resistance to herbicides that are inhibitors of acetohydroxy acid synthase (AHAS), especially the imidazolinones, will be given more detailed consideration. Resistant mutants of corn and canola were isolated by cell culture selection. Selection among mutant seed populations yielded an imidazolinone resistant wheat variety. All of these mutants contain an altered form of AHAS that is less sensitive than the normal enzyme to inhibition by imidazolinones. Mutant AHAS alleles were cloned from resistant maize and wheat lines and sequenced. Single nucleotide changes directing amino acid substitutions at positions 542 and 621 were identified. A mutant allele of the *Arabidopsis* AHAS gene was constructed by site-directed mutagenesis at codon 653, which corresponds to residue 621 of the maize enzyme. The mutant *Arabidopsis* AHAS allele (Ser653 Asn) was introduced into tobacco and potato. Resultant transgenic plants exhibit increased tolerance for applications of imidazolinone herbicides.

S10-4 THE MOLECULAR BASIS OF CELL DIVISION IN
PLANTS
D. Inzé^a, A. Hemerly^b, P. Ferreira^b, J. Engler^b, G.
Segers^b and M. Van Montagu^b

^aLaboratoire associé d'INRA and ^bLaboratorium voor Genetika, University of Ghent, Belgium.

It is now well established that the key regulatory mechanism of the cell cycle is universally conserved in all eukaryotes, including plants. Central are the so-called cyclin dependent Ser/Thr protein kinases, composed of a catalytic subunit (cdk) and a regulatory subunit (cyclin). We have characterized in the model plant *Arabidopsis thaliana* two cdks, called *cdc2a* and *cdc2b* and five cyclins, denominated *cycl1*, *cyc2a*, *cyc2b*, *cyc3a* and *cyc3b*. By whole mount *in situ* hybridizations we showed that both *cdc2a* and *cdc2b* are not only expressed in dividing cells, but also in cells which retain a competence for division. In contrast, all cyclins are exclusively expressed in dividing cells. *Cyclin1* is only expressed in G2 cells, *cyc3a* and *cyc3b* in G1 cells and *cyc2a* and *cyc2b* appear to be expressed both in G1 and G2.

To study the *in vivo* role of the two cdks, several dominant mutant forms were expressed in *Arabidopsis* and tobacco. High expression of a *cdc2a* D147N mutation is arresting cell division, where moderate expression levels lead in tobacco to plants with considerable larger cells and disturbed meristems. Despite this dramatic effect, the plants develop quite normally. This result shows that post-embryonic morphogenesis can be partly uncoupled from cell division.

S11-1 TRANSCRIPTION FACTORS GENERATING THE BLUEPRINT OF THE *DROSOPHILA* BODY PATTERN

H. Jäckle, Department of Molecular Developmental Biology, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

Segmentation during *Drosophila* embryogenesis is controlled by a cascade of zygotic gene activities that derive from polarly localized maternal factors which provide positional information along the entire longitudinal axis of the egg. The zygotic target genes can be subdivided into three distinct classes depending on the mutant phenotype and the spatial patterns of wildtype gene expression: Gap genes are expressed in broad contiguous domains that fail to develop in the corresponding gap mutant embryos; pair-rule genes are expressed in a repetitive banded pattern corresponding in position to alternating segment equivalents that fail to develop in pair-rule mutant embryos; segment polarity genes are expressed in the equivalents of anterior or posterior portions of each segment which are absent in the corresponding mutant larvae.

The cascade of zygotic segmentation genes is initiated by maternal gene products forming anterior-posterior and posterior-anterior gradients in the early embryo. After local activation in response to the maternal gene products, the gap gene expression patterns are spatially controlled by mutual interaction among the gap genes themselves. The gap-gene protein products, mostly zinc finger-type proteins, form broad and overlapping protein gradients along the longitudinal axis of the embryo. These local protein gradients contain the information for the control of zones of expression of the other gap genes which in turn provide spatial cues for generating the periodic pattern of subordinate pair-rule and homeotic gene expression.

Our results show that most of the genetic interactions within the segmentation gene cascade depend on direct DNA-protein interactions which are modulated through homo- and heterodimer formation of the gap-gene proteins that cause regulatory switches such as turning an activator into a repressor. The implication of such interactions on pattern formation in the early embryo will be discussed.

S11-3 INDUCIBLE GENE EXPRESSION IN RESPONSE TO PATHOGENS: THE ROLE OF NF- κ B

Patrick Bauerle

Abstract not received

S11-2 MEMBERS OF A LEUCINE ZIPPER PROTEIN FAMILY INVOLVED IN CIRCADIAN AND DIURNAL GENE EXPRESSION

V. Ossipow, L. Lopez-Molina, D. Talbot, D. Lavery, E. Schmidt, E. Falvey, P. Fonjallaz and U. Schibler

Department of Molecular Biology, University of Geneva, Switzerland

DBP, TEF, AND HLF are three members of a transcription factor family, PAR, that bind efficiently to sequences of the type RTTAYGTAAY. While these three proteins exhibit somewhat different spatial distribution patterns, all of them are co-expressed in parenchymal hepatocytes. In these cells, both DBP and TEF accumulate according to a circadian rhythm with a very high amplitude. The cellular HLF mRNA levels also oscillate during the day, but in contrast to the other two PAR members, this cycle is diurnal rather than circadian. In liver, transcription of several structural genes (i.e. cholesterol 7-alpha hydroxylase and HMG CoA reductase) also follow circadian or diurnal rhythms. The activity of several promoters (including the one of the cholesterol 7-alpha hydroxylase gene) is strongly enhanced by DBP, the most abundant PAR family member. Intriguingly, one of the DBP activation domains acts promoter-specifically. The role of DBP in controlling circadian gene expression is currently examined by genetic gain- and loss-of-function experiments in transgenic mice.

S11-4 MOLECULAR MECHANISMS OF RNA POLYMERASE I TRANSCRIPTION INITIATION

I. Grummt, U. Rudloff, D. Eberhard, and A. Kühn

German Cancer Research Center, Division of Molecular Biology of the Cell II, 69120 Heidelberg, Germany

Transcription initiation at the murine ribosomal gene promoter requires in addition to RNA polymerase I (Pol I) four positively acting factors, termed TIF-IA, TIF-IB, TIF-IC, and UBF. We have biochemically purified these transcription factors and have analyzed their function in preinitiation complex formation, initiation and elongation. Data will be presented showing specific DNA-protein and protein-protein interactions which lead to the assembly of productive initiation complexes at the ribosomal gene promoter. In particular, the involvement of the TIF-IB in class I promoter recognition and species-specific rDNA transcription will be discussed. We have isolated TIF-IB from Ehrlich ascites and SL1 from HeLa cells and show that they are multiprotein complexes containing TBP and three TAFs. The structure and the promoter of the human and the mouse factor as well as the interaction of individual TAFs with rDNA and with other polypeptides, i.e. UBF and TBP, will be presented.

S12-1 INTRODUCTION: PROBLEMS AND POSSIBLE MECHANISMS OF CELL PATTERNING

Tsvi Sachs, Dept. of Botany, The Hebrew University, Jerusalem 91904, Israel

A classification of major patterning mechanisms will be used to consider the cellular properties the various mechanisms require and their relations to genetic information. The spaced distribution of stomata will serve for concrete examples.

Development could be preceded by a pre-pattern. One example would be a non-random distribution of specific substances, a 'positional information', that would be expressed by cell differentiation. Another would be intracellular programs that would determine cell lineages. Such lineages can form groups of cells with specialized structures, such as stomata, in their center. Patterning requires a counting of divisions and a determination their relative orientations.

Patterning could also appear during development. These mechanisms would include cell interactions, which could be continuous and change as the cells differentiate. In one class of such mechanisms interactions would modify the relations between neighboring structures. In stomata this allows for a fine tuning of patterns formed by programmed lineages and for the induction of specialized neighboring cells. Another class of mechanisms resembles Darwinian evolution: many potential structures are initiated and order increases as a selected subclass completes differentiation. Selection by continuous comparisons between the various potential stomata are indicated in some plants by many structures that remain in different 'immature' states.

Patterning that precedes development could be directly related to controls of gene expression. However, the specification of the relative orientation of consecutive cell divisions may require interactions that are fairly distant from the genes themselves. Comparisons of alternative developing structures could be distant from gene expression and could mean that single mutations would not be expressed. Finally, different types of signals could carry patterning information and there are various ways in which they could be studied. This lecture will concentrate on descriptive methods; additional examples will be considered in the following lectures.

S12-2 FROM GENE TO FORM AND PATTERN: THE KEY BIOPHYSICAL TRANSDUCTIONS

Paul B. Green

Department of Biological Sciences, Stanford University, Stanford, CA 94305, U.S.A.

Genetic control of form and pattern must involve transductions where biochemical influence governs the configuration of solid cells and tissues. Extension of organ form, studied here as elongation, is an iterative cellular process where repeated directional expansion events are based on transverse reinforcement by cellulose and hence on transverse microtubule (MT) alignment. A dynamic MT equilibrium process, "self-cinching," is proposed to govern the degree of directionality, and thus to be the link between gene action and extension of cylindrical form. The key connection between chemical (gene) influence and organ pattern in solid tissue is proposed to be physical buckling. Non-periodic expression gradients can lead to corresponding non-periodic variation in physical (or growth) properties in a plane (e.g., the tunica). Most significantly, this non-periodic imbalance can lead to the spontaneous generation of regular periodic undulations, such as primordia in a phyllotactic pattern. This minimal energy biophysical transduction can also propagate pattern in shoots and flowers. Cell differentiation typically follows topographical change. Form and pattern appear to be expressed through key minimal energy transductions.

S12-3 PSEUDODIRECTED VARIATION IN THE RESPONSE OF CELLS TO HORMONES: A PLAUSIBLE STOCHASTIC MODEL FOR PATTERN FORMATION

F. Meins, Jr.

Friedrich Miescher Institute, P.O.B. 2543, CH-4002 Basel, Switzerland

Cells cultured from explants of tobacco leaf require exogenous cell-division factors such as the cytokinin kinetin for sustained proliferation. These cytokinin-requiring (C^-) cells give rise to cytokinin-autotrophic (C^+) variants. Some of these variants result from a meiotically transmissible change at the *Habituuated leaf-2* locus. Measurements of the rate of phenovariation show that cultured leaf cells alternate between the C^- and C^+ states at extremely high rates of $\approx 10^{-2}$ per cell generation, which are 10^2 - to 10^3 -fold more rapid than most somatic mutations in tobacco. These changes are so rapid that the classical distinction between random and induced events is blurred. Selection of alternative states arising by this form of *pseudodirected* variation results in changes that appear to be directed at the tissue level. A heuristic model will be presented to illustrate how gradients in hormone concentration could act by selection rather than by induction to give predictable patterns at the tissue and organ level. This provides a plausible explanation for the directed, but plastic nature of development in plants.

PATTERNING THE *ARABIDOPSIS* EMBRYO

S12-4 G. Jürgens, U. Mayer, M. Busch, T. Laux

Institute of Genetics, Munich University, D-80638 München, FRG

The body pattern of a flowering plant is established during two phases of the life cycle. Embryogenesis generates a relatively simple seedling pattern which consists of one linear array of a few elements along the apical-basal axis and another radial array perpendicular to the axis. In *Arabidopsis* this primary body organization is laid down in the first few days after fertilization and becomes morphologically apparent in the heart-stage embryo. At the seedling stage, the meristems of the shoot and root, which are located at opposite ends of the body axis, take over to generate the adult plant body.

Both primary pattern formation in the embryo and pattern elaboration by the shoot meristem have been genetically dissected in *Arabidopsis*. Pattern mutants are currently being used to analyze mechanisms establishing plant body pattern. Focusing on apical-basal pattern formation in the embryo, we have identified four genes involved in partitioning the axis or in directing region-specific development. The primary effects of these genes are expressed in the way the cells divide in the developing embryo. Implications for pattern formation will be discussed.

S13-1

K. von Figura

Abstract not received

S13-2 THE RESIDUES LEU (ILE)⁴⁷⁵-ILE (LEU, VAL, ALA)⁴⁷⁶, CONTAINED IN THE EXTENDED CARBOXYL CYTOPLASMIC TAIL, ARE CRITICAL FOR TARGETING OF THE RESIDENT LYSOSOMAL MEMBRANE PROTEIN LIMP II TO LYSOSOMES.

Ignacio V. Sandoval*, Juan J. Arredondo*, José Alcalde*, Alfonso González Noriega[&], Joel Vandekerckhove*, María A. Jiménez[&] and Manuel Rico[§]. *Centro de Biología Molecular "Severo Ochoa" Madrid; [†]Laboratory of Physiological Chemistry, Rijksuniversiteit Gent; [‡]Instituto de Estructura de la Materia, Madrid; [&]Instituto de Investigaciones Biomédicas, México, D.F.

LIMP II, a type II lysosomal integral membrane protein, and the CD86/LIMP II construct are targeted to lysosomes by means of a signal expressed in the tyrosine-lacking carboxyl cytoplasmic tail of LIMP II (Vega *et al.*, 1991a, 1991b). Substitution of Leu⁴⁷⁵ with Ile resulted in decreased efficiency of targeting. Mutant forms produced by substituting Leu⁴⁷⁵ by hydrophobic residues with either large (Val) or small (Ala, Gly) side chains, or by a charged residue (Asp), showed inhibited targeting. In contrast, the contiguous Ile⁴⁷⁶ residue could be replaced by either Leu, without loss in the efficiency of targeting, or by Val or Ala, with some impediment. Substitution of Ile⁴⁷⁶ by either Gly or Asp inhibited completely the targeting. Addition of the sequence SWD to the carboxyl end of the construct did not interfere with targeting. Data from ¹H NMR analysis of the icosapeptide corresponding to the carboxyl cytoplasmic tail of LIMP II indicated the predominance of structures with extended random coil conformations, suggesting that the targeting signal is contained in a domain with an extended configuration.

S13-3 RAPID INTERNALIZATION OF THE MHC-ASSOCIATED INVARIANT CHAIN AND ITS ROLE IN ENDOSOMAL TRANSPORT

Oddmund Bakke, Bjørn Bremnes, Jean Pierre Gorvel*, Toril Madsen and Espen Stang. Division of Molecular Cell Biology, Department of Biology, University of Oslo, Norway and *Centre d'Immunologie, INSERM-CNRS de Marseilles, France.

Invariant chain (Ii) is a transmembrane protein that associates with the MHC class II molecules in the ER. Two regions of the 30 residue cytoplasmic tail of Ii contain sorting information able to direct Ii to the endocytic pathway. The full length cytoplasmic tail of Ii and the two tail regions were fused to neuraminidase (NA) forming chimeric proteins (INA). By labelling the INA fusion proteins with iodinated antibody it was found that molecules with either signal were expressed at the plasma membrane and internalized rapidly. Point mutations revealed that a LI motif within the first region of the cytoplasmic tail and a ML motif in the second region was essential for efficient internalization. Invariant chain synthesized at high level in transfected cells may induce large vesicular structures. We have characterized the structures in transfected human fibroblasts as part of the endocytic pathway. Ultrastructurally, at least three morphologically distinct enlarged compartments could be discerned. These represent early and late endosomes and lysosomes. Internalization of antibodies show that invariant chain may reach the large endosomes via the plasma membrane. Internalized protein remained in the enlarged vesicles indicating an invariant chain induced delay in the pathway to lysosomes. Subcellular fractionation of metabolically labelled cells show that Ii induces a general delay in transport from early to late endosomes. From the above we may conclude that Ii induces a distinct cellular phenotype with properties that may play an essential role for proper antigen processing and intracellular MHC class II peptide loading.

S13-4 TARGETING OF MAMMALIAN GLUCOSE TRANSPORTERS

Jan W. Slot

Department of Cell Biology, Medical School, AZU - H02.314, Utrecht University, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

A family of facilitated glucose transporters, or "GLUTs" form specific pori in membranes through which glucose can diffuse into cells. Members of this family (GLUT 1,2,3...) are expressed in different cell types and, in spite of a high degree of amino acid and structural homology, they have specific signals that target them to different cellular membranes. GLUT1, which is expressed in many cultured cell lines, is in polarized cells most prominent in the basolateral domain of the plasma membrane. GLUT2 has been described to be enriched in specialized parts, the microvilli area, of the plasma membrane of pancreatic β -cells (1). We have localized GLUT3 in the apical plasma membrane of polarized cells. GLUT4, which is expressed in muscle and adipose tissue, has a dual cellular distribution. In basal cells it resides intracellularly in trans Golgi elements and in endosome related small vesicles and tubules. After insulin stimulation a significant part of the GLUT4 molecules is translocated to the plasma membrane. Its presence in clathrin coated pits and early endosomes suggests that GLUT4 continuously circulates in a plasma membrane - endosome pathway as long as the insulin stimulation lasts.

The GLUT proteins provide an ideal system for studying the molecular regulation of membrane protein targeting. Chimeric proteins of the different isoforms can be constructed while maintaining the overall structure of the protein. By transflecting these into cells it is possible to identify the targeting domains on the molecules (2, 3). (1) L. Orci *et al.*, 1991, Science (Wash.D.C.) 245: 295-7. (2) D.E. James *et al.*, 1993, J.Cell Sc. 104:607-612. (3) D.E.James *et al.*, 1994, Trends Cell Biol. 4:120-126.

S14-1 THE T CELL RECEPTOR: A COMPLEX STRUCTURE TRIGGERS COMPLEX INTRACELLULAR SIGNALS

Paul Burn,
Hoffmann-La Roche Inc., Nutley, NJ 07110, USA

The response of T lymphocytes to antigen is mediated by a multisubunit cell surface receptor complex involving the T cell antigen receptor (TCR)/CD3 complex and co-receptors such as CD4 and CD8. Engagement of this complex initiates a cascade of biochemical events that result in multiple intracellular signaling events including the activation of protein tyrosine kinases and protein tyrosine phosphorylation. Depending from the stimulus, this eventually leads to cellular activation, altered gene expression, the onset of replication, proliferation and differentiation of the triggered cell. The exact molecular mechanisms of how signals are transduced across the cellular membrane and into the nucleus have not yet been unraveled. The rapid activation of *Src* family protein tyrosine kinases and the tyrosine phosphorylation of a distinct set of cellular proteins, however, seem to be crucial to the signaling process. Here, we report on the identification of the kinases involved in this signaling cascade, their regulation, and the search for potential substrates. Biochemical evidence is provided for a function for *Src* homology 2 (SH2) domains in the intramolecular and intermolecular regulation of *Src* family protein tyrosine kinases catalyzed by the protein tyrosine phosphatase CD45 and the protein tyrosine kinase Csk. The discussed results support the view of an involvement of *Src* family kinases in linking antigen receptor complex-mediated signaling pathways with *Ras*-mediated signaling pathways.

S14-2

INTERACTION OF cGMP AND Ca⁺⁺ SIGNAL TRANSDUCTION PATHWAYS

U.Walter, E.Butt, T.Jarchau, J.Geiger, S.M. Lohmann, C. Nolte, and M.Meinecke

The NO/cGMP signal transduction system has recently emerged as an important pathway involved in the regulation of intracellular Ca⁺⁺ and Ca⁺⁺-dependent functions (1). The structure and function of some of the major components of the NO/cGMP signal transduction system (NO synthases, receptor-linked and soluble guanylyl cyclases, cGMP effector systems such as cation channels, phosphodiesterases and protein kinases) will be briefly reviewed. Evidence has accumulated that the cardiac L-type calcium channel is inhibited by cGMP mediated by cGMP-regulated protein kinases and phosphodiesterases. In human platelets, stimulation of the cGMP-dependent protein kinase inhibits the agonist evoked activation of phospholipase C, protein kinase C and myosin light chain kinase. Further evidence suggests that the cGMP-dependent protein kinase inhibits the mobilization of Ca⁺⁺ from intracellular stores and the store-associated secondary calcium influx without affecting the rapid phase of ADP-evoked calcium influx. Data obtained with human platelets deficient in cGMP-dependent protein kinase substantiate the important role of this kinase in calcium regulation. Other experiments suggest that activation of the cGMP-dependent protein kinase also inhibits the thapsigargin-evoked Ca⁺⁺ release from intracellular stores. During coincubation of platelets and endothelial cells, endothelial-derived factors inhibit the platelet calcium response mediated by both cGMP- and cAMP-dependent protein kinases. The recent cloning of some components of the NO/cGMP signal transduction system allows now the investigation of the interaction of the cGMP and Ca⁺⁺ signal transduction systems at the molecular level.

1) Schmidt, H.H.W., Lohmann, S.M., and Walter, U. (1993) *Biochim. Biophys. Acta* 1178, 153-175

S14-3

G PROTEINS INVOLVED IN THE CALCIUM CHANNEL SIGNALLING

J. Hescheler, F. Kalkbrenner, V.E. Degliar, B. Wittig^a and G. Schultz

Institut für Pharmakologie (Thielallee 69/73), ^aInstitut für Molekularbiologie und Biochemie (Arnimallee 22), Freie Universität Berlin, D-14195 Berlin, FRG

In order to identify the heterotrimeric G proteins involved in the inhibition of Ca channels by various receptors, we injected anti-sense oligonucleotides hybridizing to the mRNAs encoding the α , β and γ subunits of G proteins into the nuclei of pituitary cells of the line GH₃. Using this system, we previously demonstrated the involvement of $\alpha_{\text{G}1}\beta_{\text{G}3}\gamma_{\text{G}4}$ and $\alpha_{\text{G}2}\beta_{\text{G}1}\gamma_{\text{G}3}$ in the inhibition of L-type Ca channels by acetylcholine and somatostatin, respectively. In order to investigate whether the same triplets may be involved in inhibitory effects by other receptors we determined the G proteins coupling galanin receptors to L-type Ca channels. After injection of $\alpha_{\text{G}1}$ antisense oligonucleotides, inhibition of Ca currents was reduced from 20% in control injected cells to 5% in anti- $\alpha_{\text{G}1}$ injected cells. Injection of β_2 and β_3 antisense oligonucleotides decrease the Ca current inhibition to 10% and 15%, respectively. Injection of γ_2 and γ_4 antisense oligonucleotides decreased it to 10% and 12%, respectively. Corresponding experiments in pancreatic B-cells of the line RINm5F revealed similar combinations of antisense effects. Our data suggest that the inhibition of L-type Ca channel by galanin is mediated by two heterotrimeric G proteins, mainly by $\alpha_{\text{G}1}\beta_{\text{G}2}\gamma_{\text{G}4}$ and less efficiently by $\alpha_{\text{G}1}\beta_{\text{G}3}\gamma_{\text{G}4}$, a combination which is also used by muscarinic M₄ receptors in these cell lines.

S14-4

INTRACELLULAR Ca²⁺ STORES: STRUCTURE AND FUNCTION

Jacopo Meldolesi

Abstract not received

S15-1 REGULATION OF CELL GROWTH AND DIFFERENTIATION BY REVERSIBLE TYROSINE PHOSPHORYLATION

Axel Ullrich

Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany

Signals mediated by protein tyrosine kinases (PTKs) are of crucial importance for a variety of fundamental biological processes such as cell growth, hematopoietic cell differentiation, formation and maintenance of the nervous system, the establishment of the vascular system, and the regulation of cellular immune responses. Recent advances have demonstrated that cellular proteins with partial homology to specific regions in the pp60^{c-src} proto-oncoprotein, designated SH2 and SH3 domains, represent primary signal transducers which activate diverse signaling pathways within the cell upon interaction with autophosphorylated PTKs. This interaction involves PTK phosphotyrosine residues, including short flanking sequences and the SH2 domain(s) of the signal-transducing factor, and may result in tyrosine phosphorylation of the latter. The characteristics of the signal and therefore the cellular response appear to be defined on one hand by the differential affinity of phosphotyrosine binding sites for different primary signal transducers of one PTK relative to another and on the other hand by the repertoire and concentration of such factors presented by the cell. Imbalances in this system due to over/underexpression or mutation of individual elements in these signaling pathways can result in pathophysiological disorders such as cancer and diabetes.

Various approaches towards the elucidation of phosphotyrosine-mediated signaling mechanisms and their biological and pathological relevance will be discussed. Furthermore, recent results regarding the relevance of protein tyrosine phosphatases for the PTK signaling process will be presented.

S15-2

ErbB Receptor Tyrosine Kinases: Signal Transduction and Oncogenesis

Yosef Yarden, The Weizmann Institute of Science, Rehovot 76100, Israel.

The sub type I of receptor tyrosine kinases includes four transmembrane glycoproteins whose prototype the receptor for epidermal growth factor (EGF). The interest in this family of receptors arose because members of this group have been implicated, more than other growth factor receptors, in the development of several human adenocarcinomas. For example, overexpression of ErbB-2 (also called Neu or HER-2) has been correlated with poor prognosis of several types of human adenocarcinomas. A rodent oncogenic form of ErbB-2 contains a point mutation, that results in constitutive activation of the kinase and a consequent permanent coupling to cytoplasmic signaling pathways. This is mediated by five C-terminally located tyrosine autophosphorylation sites. By using site-directed mutagenesis we found that the most carboxy terminal site (Tyr1253) is sufficient to confer oncogenicity, by coupling to an intracellular pathway that includes Ras, MAP-kinase and transactivation of c-Jun.

The search for ligands that activate tyrosine phosphorylation of ErbB-2 led to the isolation of a 44-kDa glycoprotein, termed Neu differentiation factor (NDF, or heregulin). We identified twelve isoforms of proNDF, whose structural variation results from tissue-specific alternative splicing. One group of isoforms is enriched in mesenchymal cells, while the other is confined to neuronal tissues. Members of the latter group function as astroglia-specific survival and maturation factors. By using recombinant soluble extracellular domains of the four ErbB receptors we found that NDF binds with high affinity to ErbB-4 and with lower affinity to ErbB-3. Heterodimers that contain ErbB-2 and one of the NDF receptors exist, and they may differ in ligand affinity and signaling properties. This observation implies that in addition to the classical vertical signaling pathway of each ErbB molecule, lateral receptor-receptor interactions may diversify the mechanism of signal transduction by the ErbB family.

S15-3 THE HGF RECEPTOR AND ITS LIGAND: STRUCTURE, SIGNAL TRANSDUCTION AND BIOLOGY

P.M. Comoglio Department of Biomedical Sciences, University of Torino School of Medicine, c.so M. D'Azeglio 52, Torino (Italy).

Hepatocyte Growth Factor (HGF) is a powerful mitogen, motogen and morphogen for epithelial and endothelial cells. The HGF α chain binds with high affinity to a tyrosine kinase receptor, p190^{MET}, encoded by the MET proto-oncogene.

HGF binding triggers tyrosine autophosphorylation of the receptor β subunit; phosphorylation on the major phosphorylation site (Y¹²⁵) upregulates the receptor kinase activity, increasing the V_{max} of the phosphotransfer reaction. Negative regulation of the receptor kinase activity occurs through exogenous phosphorylation of a unique serine residue in the juxtamembrane domain (S⁹⁵).

Upon ligand binding, the HGF/SF receptor recruits and activates several cytoplasmic effectors, including the Shc and GRB-2 adaptors, PI 3-Kinase, PLC- γ , pp60^{c-src}, a tyrosine phosphatase, and a Ras-guanine nucleotide exchanger. Interaction with these SH2 containing effectors is mediated by phosphorylation of a single multi-specific binding site, consisting of a pair of tyrosines (Y¹³⁴ and Y¹³⁶) located in the C-terminal tail. Mutation of the two tyrosines results in loss of biological function, as shown by the abrogation of the transforming activity in the oncogenic counterpart of the receptor.

HGF induces a pleiotropic response involving expression of a set of specific genes controlled at transcriptional level. These include genes encoding transcriptional factors, proteolytic enzymes involved in cell growth and motility and the HGF receptor itself (c-MET).

S15-4 NEUROTROPHINS AND THEIR RECEPTORS

Y.-A. Barde
Department of Neurobiochemistry Max-Planck Institute for Psychiatry 82152 Martinsried Germany

During the development of the vertebrate nervous system, many cells are eliminated when long lasting relationships are first established, for example when neurons contact their targets, or oligodendrocytes their axons. It is becoming apparent that cell numbers can be regulated by the limited availability of factors belonging to a gene family -the neurotrophins. Thus, nerve growth factor (NGF) prevents the death of neural crest-derived neurons, and brain-derived neurotrophic factor (BDNF) saves motoneurons both during development and after axotomy. But recent results indicate that neurotrophin-3 (NT-3) regulates neuronal numbers *in vivo* long before normally occurring cell death is seen in NT-3-responsive ganglia. Already during gangliogenesis, the limited availability of NT-3 regulates neuronal proliferation and/or the differentiation of neuronal progenitor cells. In addition, a direct mitogenic effect of NT-3 has been demonstrated on oligodendrocyte precursor cells. Neurotrophins mediate their effects at least in part following binding to and activation of one of the 3 members of a receptor tyrosine kinase family expressed in the nervous system -the trks. The exact role played by another membrane protein, the low affinity NGF receptor (or p75), that binds all known neurotrophins with similar affinities (unlike the trks) remains an interesting enigma. While this protein might modulate the affinity and the specificity of binding of the trks, a direct association of both proteins has been difficult to demonstrate.

FLOW SORTING OF SINGLE CHROMOSOME TYPES IN THE STUDY OF PLANT GENOME

Mo-1

J. Doležel^a, J. Macas^b, S. Lucretti^c, P. Samec^b, J. Čihálíková^a and I. Schubert^d

^aInstitute of Experimental Botany, Olomouc, Czech Republic;

^bInstitute of Plant Molecular Biology, České Budějovice, Czech Republic; ^cENEA, C.R.E. Casaccia, Rome, Italy; ^dInstitute of Plant Genetics, Gatersleben, Germany

Flow cytometric sorting permits isolation of single chromosome types in large quantities. The suitability of sorted chromosomes for construction of chromosome-specific DNA libraries and for gene mapping has been well documented in human. Unfortunately, the application of the method to plants has been significantly delayed.

We have developed an original procedure for preparation of chromosome suspensions in several economically important plants (field bean, pea, maize). Chromosome suspensions were analysed flow cytometrically and single chromosome type fractions were sorted. Low number of sorted chromosomes (100 - 200) was sufficient for polymerase chain reaction. The technique was successfully applied to localisation of five seed storage protein genes in field bean. The use of translocation lines improved the resolution of single chromosome types and permitted subchromosomal localisation of DNA sequences. RAPD with sorted chromosomes revealed genomic distribution of fragments generated with total DNA as a target. It was also possible to observe moderate differences of RAPD patterns among individual chromosome fractions.

The work is in progress with physical mapping of other DNA sequences and with the construction of chromosome-specific DNA libraries.

SUPRACHROMONEMAL NETWORK: RECONSTRUCTION DURING CHICK EMBRYO CHONDROCYTE DIFFERENTIATION

Mo-3

J.e. Erenpreisa, A.Zhukotsky*

Latv. Inst. of Exp. and Clin. Med., Riga, Latvia;
* Res. Inst. Physico-chemical Med., Moscow, Russia.

The method of computational image analysis of the chromatin network in interphase nuclei of the DNA-stained cell imprints and ultrathin sections was elaborated and applied to developing chondrocytes in chick limb buds.

The Rabl orientation of the chromosomes with associations of pericentromeric heterochromatin and NORs was found to provide a stable framework of the net, persisting throughout differentiation process.

Small peripheral alveoles, enclosed by intra- and interchromonemal links of intercalary heterochromatin, construct the dynamic part of the network. They appear simultaneously with the chromatin decondensation in differentiating chondroblasts and disappear in terminally differentiated chondrocytes prior to apoptosis.

The pattern of the chromatin compaction and related rearrangement of the chromatin network in terminally differentiated chondrocytes resembles that occurring during metaphase plate formation in mitotic chondroblasts.

STACKING STRUCTURE OF TRIVALEINE-TRINUCLEOTIDE COMPLEXES

Mo-2

S. Streltsov, L. Martinkina,
T. Semenov, Yu. Vengerov
Engelhardt Institute of Molecular Biology, Moscow, Russia

Fluorescence, linear dichroism and electron microscopy showed that complexes of trivaline β -dimer with ds and ss nucleic acids are able to compact not only in a plane perpendicular to the sugar - phosphate frame direction but in the same direction too. Homogeneous in thickness (~160-180 Å), extended structures 1000 Å in length are formed from trivaline and trinucleotides (hypoxanthine, homopyrimidine, of random nucleotide composition; of desoxy- or ribo- row; 5'-phosphorylated or not). The considerable fluorescence of them indicates the highly ordered nucleotide arrangement in them.

It was shown that addition of condensing agents to the solution of trivaline complex with 5'-pdGTT leads to formation of nucleic oligomers up to 12 bases in length. In the absence of peptide the ~100 times more concentration of trinucleotides is required for the oligomerization.

The model was proposed for the formation of compact structures, containing trivaline and nucleic acids (ds and ss) of random length.

So, β -dimer of trivaline in such conditions can appear: (1) as a model of DNA-ligase functioning on the stage of enzyme-substrate complex formation; (2) as a probable evolutionary precursor of these enzymes; (3) as a source of preenzymatic synthesis of long random sequences of nucleic bases, from which the biologically considerable ones were chosen in evolution.

MAPPING SITES OF DNA CLEAVAGE
in vivo BY TOPOISOMERASE II IN THE HUMAN HPRT GENE REGION

J. Fajkus^a, J.A. Nicklas^b and R. Hancock^c

^aInstitute of Biophysics, Czech Acad. Sci., Brno 61265, Czech Republic; ^bVermont Cancer Centre, Burlington, VT 05401, USA; ^cCentre de Recherche en Cancérologie de l'Université Laval, Québec, Canada G1R 2J6

We have mapped sites of DNA cleavage mediated by topoisomerase II *in vivo* in a 2.5 Mb region around the human HPRT gene in Xq26, using DNA fragments resulting from VM-26-stimulated topoisomerase II cleavage followed by restriction with rare cutting enzymes. Current evidence indicates that topoisomerase II and its target sequences are localised at nuclear matrix attachment regions (MARs), and we believe that our map therefore shows the borders of DNA domains.

Topoisomerase II cleavage sites were detected by indirect end-labelling, hybridising probes available for this region to blots of PFGE gels and subtraction of bands produced by restriction alone. However, the only MAR mapped in this region by *in vitro* methods (in the first intron of the HPRT gene) was not detected by our *in vivo* approach. Our results are discussed with respect to the positions of deletion and recombination breakpoints, to evaluate the hypothesis that certain breakpoints could result from the action of topoisomerase II at MARs. [Supported by American Cancer Society and MRC of Canada]

Mo - 5

ULTRASTRUCTURAL LOCALIZATION OF
HAEIII TARGET SEQUENCES ON HUMAN
CHROMOSOMES BY RE/NT WITH COLLOIDAL
GOLD

R. Del Coco^a, C. Cinti^b, S. Santi^a, M.C. Maltarello^b,
L. Stuppi^a

^a Istituto di Citomorfologia Normale e Patologica del C.N.R.,
CHIETI e BOLOGNA

^b Istituto di ricerche "Codivilla-Putti", c/o Istituti Ortopedici Rizzoli,
BOLOGNA

The induction of nicks by restriction enzymes (RE) in human chromosomes used as starting sites for the incorporation of labeled precursors by "in situ" nick-translation (RE/NT), produces a banding pattern peculiar for each restriction enzyme.

The digestion with HaeIII (GGGCC recognition sequence), followed by "in situ" NT, produces a C-negative banding (1), (2), or a C-positive banding (2), after short time or long time of digestion, respectively. When NT experiments are performed with Digoxigenin-UTP incorporation and labeled precursors revealed with 5 nm gold-antidigoxigenin MoAb, the punctate and strong electron-density of colloidal-gold particles observed in electron-microscopy reveals a detailed localization of cleaved target sequences, but does not produce banding pattern.

References:

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- 2) Sumner, A.T., Taggart, M.H., Mezzanotte, R., Ferrucci, L. 1990. Patterns of digestion of human chromosomes by restriction endonucleases demonstrated by "in situ" nick-translation. *Histochemical Journal*, 22:639-652.

Mo - 7

DNA REPLICATION SITES IN NUCLEI OF
PISUM SATIVUM

E. Sparvoli^a, M. Levi^a, S. Citterio^a, B. Gabara^b,
K. Bianco^a and E. Rossi^a

^a Department of Biology, University of Milan, Italy; ^b
Department of Plant Cytology and Cytochemistry, University of
Lodz, Poland

When nuclear DNA replication is evidenced by immunofluorescence after bromodeoxyuridine labelling, DNA replication sites appear as finely distinct fluorescent spots representing replication clusters. Three-day-old pea seedlings were labelled with a pulse of bromodeoxyuridine and then transferred to water for chase periods of different lengths in order to follow the fate of labelling through at least two or three cell cycles. When the immunofluorescent reaction was performed on nuclei isolated from meristematic and differentiating cells of the roots, the labelling was distributed in specific territories of the nucleus, whereas other territories were completely devoid of fluorescence. The same analysis was performed on root sections, in order to verify whether adjacent cells were complementary labelled, as could be expected due to chromosome segregation after DNA replication in the absence of the labelled precursor.

Furthermore, the distribution of bromodeoxyuridine labelled sites was compared with that of an anti PCTAIRE antibody to study the possible involvement of cdc2-related proteins in G1/S transition.

Mo - 6

EVIDENCE FOR A SEQUENCE-DIRECTED CON-
FORMATION PERIODICITY IN THE GENOMIC
HIGHLY REPETITIVE DNA, DETECTABLE
WITH SINGLE-STRAND-SPECIFIC CHEMICAL
PROBES

R. Matyášek, B. Gazdová and M. Bezdečk

Institute of Biophysics, Academy of Sciences of
the Czech Republic, 612 65 Brno, Czech Republic

The single-strand specific chemical probes, potassium permanganate ($KMnO_4$), diethylpyrocarbonate (DEPC) and osmium tetroxide complexes with pyridine (OsO_4 -Py) and bipyridine (OsO_4 -BiPy) have been used to study the local sequence-dependent conformation polymorphisms of the repetitive DNA sequence HRS60 of *N. tabacum* at the level of the single base pair and dinucleotide step. The local structures sensitive to the probes specific to thymine residues ($KMnO_4$, OsO_4) have the 183 bp periodicity, characteristic for HRS60 monomeric units, and are detectable in genomic DNA *in vitro*, *in situ* and in recombinant lambda phage DNA containing the long tandem of HRS60 sequences. Their position with respect to known conservative restriction sites and with respect to individual complementary strands were studied. Using lower reaction temperatures or the presence of the distamycin A in the reaction mixture increased the sensitivity of these sites to the probes used. The conformation polymorphisms of individual HRS60 monomeric units was detected also on the basis of different electrophoretical mobilities.

Mo - 8

INSTABILITY OF THE GENOME AND HYPERMUTA-
BILITY OF PERIPHERAL BLOOD LYMPHOCYTES
IN PATIENTS WITH DYSPLASTIC PIGMENTED
NAEVI AND MALIGNANT MELANOMAS

K.P. Ganina, S.S. Kireeva

Laboratory of Genetics,
Kavetsky Institute of
Experimental Pathology,
Oncology and Radiology,
Academy of Sciences of
Ukraine, Kiev

Increase in rate of chromosome aberrations and sisters' chromatid exchanges per cell and in number of such cells was registered in lymphocytes' culture in our cohort of patients with dysplastic pigmented nevi and malignant melanomas. Hypermutability of lymphocytes with respect to mitomycin "C" of our cohort in comparison with data derived from general population was also observed. It was discussed the role of genome instability in biology of somatic cells and their transformation.

Mo - 9

USE OF SCANNING CYTOSPECTROPHOTOMETRY FOR ANALYSIS OF INTERPHASE NUCLEI OF LYMPHOCYTES IN PERIPHERAL BLOOD OF PATIENTS WITH BREAST CANCER AND UTERINE CERVIX CANCER

L. Naleskina , L. Isakova

Laboratory of Genetic R.E.Kavetsky Institute of Experimental Pathology,Oncology and Radiobiology Academy of Science of Ukraine ,Kiev

Structural density peculiarities of chromatin interphase nuclei of lymphocytes of the peripheral blood (LPB) from control women,patients with precancerous breast and uterine cervix and patients with breast cancer (BC) and uterine cervix cancer (UCC) with registration of the degree malignant tumour was investigated.The estimate of the optical density chromatine of the interphase nuclei LPB was conducted by means automatic device IBAS (Germany) and MCFU (Russia).LPB of the patients with BC and UCC have different structural density peculiarites of the chromatine by comparison with lymphocytes of the control women,precancerous breast and uterine cervix.

Mo - 11

PECULIARITIES OF THE PRIMARY STRUCTURE OF DNA OF THE CHINESE HAMSTER SYNAPTONEMAL COMPLEX

O.I.Karpova, M.V.Penkina, S.Ya.Dadashev,
N.V.Mil'shina, H.E.Fernandes and Yu.F.Bogdanov

Vavilov Institute of General Genetics Russian Academy of Sciences, Moscow, Russia

DNA of Chinese hamster synaptonemal complex (SC) has been cloned and partially sequenced. Computer analysis revealed similarities and differences between the sequences of SC DNA and those of separate families of eukaryotic genomic DNA. SC DNA has homology with LINE-family and in contrast to genomic DNA is enriched with alternative (GT/CA)n sequences and palindromes. TGT/ACA and GTG/CAC triplets with periodicity of two nucleotides occur mainly in SC DNA of hamster and rat as well as in DNA of microsatellite loci of human chromosome 13 but not in nuclear matrix-attached regions of DNA (MARs). By its ability to form potential hairpin loops SC DNA is similar to MARs and differs substantially from DNA of microsatellite loci. It is noteworthy that SC DNA, which is supposedly involved in meiotic recombination, and DNA of immunoglobulin genes, involved in somatic recombination, are both enriched with palindromes. The discovered peculiarities of the structure of hamster SC DNA allow to separate it into a special family of genomic DNA sequences.

Mo - 10

USE OF SCANNING CYTOSPECTROPHOTOMETRY FOR ANALYSIS OF DNA CONTENT IN BUCCAL MUCOSA OF BREAST CANCER PATIENTS

N. Boroday

Laboratory of genetic R.E.Kavetsky Institute of Experimental Pathology,Oncology and Radiobiology Academy of Science of Ukraine, Kiev

The quantitative method for analyzing nuclear image gives more reproducible results because DNA measurement of patients with malignant disease are very perspective.

We established the increase of DNA nuclear content of the cells in spinous layers of the buccal mucosa in patients with tumours which are in distant from the oral cavity (breast cancer) in comparison with benign. This results suggested that some changes of DNA content in epitheliocyte nuclei and index nuclei heterogeneity in DNA content can be indicated by hormonal breakround in the organizm and by the level of disturbances in maturation and differentiation of cells to the spinous layer of the oral mucosa of breast cancer patients.

Mo - 12

A NUCLEAR TERRITORY DURING INTERPHASE
H.R. Junéra, C. Masson, G. Géraud and D. Hernandez-Verdun. Institut Jacques Monod, 2 place Jussieu, 75252 Paris Cedex 05 France

The 3-D architecture of the ribosomal genes (rDNAs) was studied during G₁, S and G₂ phases in PtK₁ cells and compared with the 3-D distribution of proteins related with ribosomal activity, the RNA polymerase I transcription factor UBF and Ag-NOR proteins. The S phase is characterized by the presence of PCNA antigens detected by immunofluorescence, and by the distribution of ribosomal genes detected by *in situ* hybridization. Both labellings are performed in the same cells making it possible to colocalize genes and proteins in 3-D by confocal microscopy. On optical sections, we observed foci in which rDNA and PCNA labelling are colocalized and others with rDNA but without PCNA labelling. This indicates that replication occurs at different sites simultaneously for the rDNAs of one chromosome. By merging all the optical sections, it appears that all the rDNAs are within the nucleolus and are distributed in alternate regions of gene accumulation (intense spots) linked by weak fluorescent threads. The number of intense spots increases during S phase and their organization is more relaxed in S and G₂ than in G₁ phase. A similar distribution was observed for the Ag-NOR proteins whose distribution has been recorded by reflection imaging mode and for antibodies directed against UBF. Colocalization of UBF and rDNAs was observed in intense spots and relaxed threads, but rDNA spots can be observed without UBF associated. This can be interpreted as different sites of active and inactive genes in one NOR. By electron microscopy, using serial sections and computer 3-D reconstruction, fibrillar centres (FCs) formed discrete structures (about 10 in G₁ and 20 in G₂ phase) with loops of dense fibrillar component connecting the different FCs. The size and 3-D arrangement of the FCs in G₁ and G₂ are similar to the intense spots of rDNAs observed in 3-D reconstruction by confocal microscopy.

Mo-13 NUCLEAR BODIES IN A HIBERNATING DORMOUSE. A CYTOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDY

M. Malatesta^a, C. Zancanaro^{a,b}, P. Vogel^c, S Fakan^a

^aCenter of Electron Microscopy, Lausanne, Switzerland; ^bInstitute of Human Anatomy and Histology, Verona, Italy; ^cInstitute of Zoology and Animal Ecology, Lausanne, Switzerland.

Brown adipocyte and liver cell nuclei of the dormouse *Muscardinus avellanarius* were studied in hibernating, arousing and euthermic individuals by means of ultrastructural cytochemistry and immunocytochemistry.

Brown adipocyte and liver cell nuclei showed a similar general morphology in the three groups. However, hibernating individuals had peculiar structural constituents in the nucleus namely, coiled bodies (CBs), often in contact with the nucleolus, and amorphous bodies (ABs). Bundles of nucleoplasmic fibrils (NF) were only present in brown adipocyte nuclei of hibernating animals. In arousing animals only some poorly structured CBs were found. None of these nuclear structural constituents was found in nuclei of euthermic individuals.

Cytochemical and immunocytochemical results showed that CBs contained p80 coilin and both nucleoplasmic and nucleolar antigens. ABs contained RNA and nucleoplasmic ribonucleoproteins. A possible involvement of CBs and ABs in storing and/or processing RNA which must be rapidly and abundantly released at the arousal is suggested. NF did not contain nucleic acids or ribonucleoproteins and were apparently made of proteinaceous material. Their functional role in the nuclear metabolism of brown adipocyte during hibernation remains unclear.

CHROMOSOME INSTABILITY

Mo-15 IN ENDOMETRIAL CARCINOMA PATIENTS
L. Polichshuk, I. Nesina, L. Buchinskaya
 Laboratory of Genetic, Institute of Experimental Pathology, Oncology and Radiobiology, Acad. Sci. of the Ukraine, Kiev, Ukraine

There had been investigated the level of chromosome aberrations, polymorphism of heterochromatic regions on chromosome 1, 9 and 16 and ribosomal gene activity in cultured blood lymphocytes in patients with endometrial cancer.

The frequency of chromosome aberrations reliable increased up to 5,21±0,53% compared with 0,67±0,21% in control. C-band variability (including extremal variants and heteromorphism homologues) in cancer patients was differed from that in health woman. It was found increasing number of active NOR's and frequency of acrocentric chromosome association in patients with cancer. All indices were more expressive in endometrial cancer patients from the families with tumour pathology in pedigrees.

EXTRACHROMOSOMAL PERIPHERAL MATERIAL IN WHEAT ENDOSPERM

**Mo-14 V.Burakov, I.Chaban, V.Polyakov,
Y.Chentsov**

Dept. of Cytology and Histology, Biological Faculty, Moscow State University, Moscow 119899, Russia

Extrachromosomal peripheral material (EPM) or matrix around mitotic chromosomes reveals by electron microscopy on all stages of mitosis of wheat endosperm. Very sharply this EPM layer can be seen in metaphase and anaphase that differes wheat endosperm chromosomes from other plant objects. EPM covers as a muff each chromosome and does not enter inside chromosome body. EPM has spongy-like crumby structure and consists mainly of ribosome-like granules and different fibres positively stained by Bernhard method. The clear parallelism between changes of nucleoli and development of EPM can be observed, that take the possibility to consider that the structure of EPM contain the material of nucleolar and extrachromosomal origin. In telophase it is seen the opposite inclusion of EPM in the structure of newly organized nucleoli.

CHROMATIN STRUCTURE OF NICOTIANA TABACUM CHROMOSOME ENDS

Mo-16 A. Kovářík, J. Fajkus and M. Bezděk

Institute of Biophysics, Academy of Sciences, Královopolská 135, 612 65 Brno, Czech Republic.

In most higher eucaryots the chromosome ends are composed of highly conserved telomeric DNA repeats and less conserved, subtelomeric repeats. To determine the length and structure of telomeric regions in *Nicotiana tabacum* we have applied PFGE and molecular hybridization using telomeric (TTAGGG)_n and highly repetitive HRS60 DNA probes. Sequential *in situ* hybridization previously showed co-localization of both probes to chromosome ends. Using restriction enzyme analysis and *Bal*31 nuclease digestion we show that telomeric repeats of *Nicotiana tabacum* occupy in average 50kb of each chromosome end. The tandemly arranged, species specific HRS60 repetitive sequences were found to be organized in clusters of at least 50kb with apparently no intervening sequences. Using PFGE we detected fragments hybridizing to both telomeric and HRS60 probes indicating a close proximity of both loci. Frequently cutting enzymes showed the presence of a short spacer region linking both clusters. Micrococcal nuclease digestions revealed periodicity in the DNA protection in isolated nuclei suggesting the nucleosomal structure of the telomeric chromatin.

CONSERVATION AND DIVERGENCE OF DNA SEQUENCES
IN PLANT GENOMES

Mo-17 R.B. Flavell

John Innes Centre, Norwich Research Park,
Colney, Norwich, NR4 7UH, UK

The architecture of a genome can be understood much better if the functions and characteristics of its constituent sequences are identified. The sequences of telomeres and coding sequences are highly conserved. Most of the repeated sequences are not highly conserved, in sequence, number or position. Thus genomes consist of sequences evolving at very different rates. The repeated sequences include tandem arrays of units and various kinds of transposable elements. Turnover of the repeated sequences accounts for much of the structural diversity between genomes within species and between related species.

In spite of the structural diversity conferred by the behaviour of families of repeated sequences, the linear order of genes is frequently conserved within large chromosome segments. This discovery, greatly endorsed by the most recent molecular mapping results, provides new understanding on the architecture of genomes and new approaches for the identification and isolation of genes.

The principles defined in this abstract will be illustrated by special reference to plant genomes.

FUNCTIONS OF THE v-Src PROTEIN TYROSINE KINASE

Mo-18

V. Fincham, M. Frame, B. Haefner, A. Wyke
and J. Wyke

The peripheral non-receptor tyrosine kinase oncogene, v-Src, has pleiotropic effects on both cell growth and cell behaviour. It is a potent mitogen for quiescent cells, substituting for both competence and progression factor-mediated signals but, unlike growth factors, it also induces cellular morphological transformation. These effects of v-Src reflect the separate but complementary activities of its catalytic domain, the SH2 and SH3 domains that dictate its interactions with other proteins and the amino terminal myristylation motif that determines subcellular localization. We are dissecting the activities of v-Src by studying mutant proteins, including those with temperature sensitive (ts) effects, in different cellular backgrounds and with the following results.

Activation of a ts v-Src kinase rapidly increases activity of both the transcription factor, AP-1, and MAP kinase, an enzyme that could enhance AP-1 activity by both phosphorylation of c-Jun and increased c-fos transcription; the relative contribution of these two events depends on the cells in which v-Src is expressed. Transient early AP-1 activation requires proper location of v-Src at the cell periphery and it is essential for mitogenesis. It is not, however, sufficient for entry into S-phase, there being a second need for v-Src later in G₁. Morphological transformation by v-Src does not require AP-1 activation but, in its initial stages at least, seems linked to events at the cell periphery, notably phosphorylation of proteins that bind to the v-Src SH3 domain such as paxillin and the p85 subunit of PI-3 kinase.

COOPERATIVE PROCESSING OF EXTRACELLULAR INFORMATION BY PROTEIN-TYROSINE KINASES AND CELL ADHESION MOLECULES

Mo-19 Michael Kraus and Bernhard Wolf

AG Medizinische Physik und Elektronenmikroskopie,
Institut für Immunbiologie, Albert-Ludwigs-Universität Freiburg,
Stefan-Meier-Str. 8, 79104 Freiburg i. Br., FRG.

Activation of receptor tyrosine kinases by multiple growth factors (GFs) is a major process through which mitogenic information is transmitted into cells. Cell cycle progression additionally requires the coordinated interaction of cellular adhesion receptors with extracellular matrix (ECM) molecules. Recent data from several groups, which demonstrated that integrin-ECM contacts promote multiple phosphorylations of intracellular components although the integrin cytoplasmic domains do not have intrinsic protein kinase activity, support the theory that some adhesion receptor families transduce extracellular signals cooperatively with protein-tyrosine kinases (PTKs). Based on the well established assumption that adhesion receptors induce an aggregation of PTKs through a rearrangement of the cytoskeleton, a mathematical minimal model for the regulation of PTKs is presented which accounts for the synergism between different environmental signals mediated by GFs and cell adhesion molecules (CAM) or cell adhesion associated substances like carcinoembryonic antigen (CEA). The model, which is closely related to a two-dimensional Ising model describing order-disorder transitions in ferromagnetic crystals, provides evidence that a cell-type-specific pattern of CAMs may function as a molecular amplifier which promotes the metastatic activity of malignant tumor cells through the indirect induction of intracellular PTK activity.

Reference: M. Kraus and B. Wolf: Ising model for cooperative processing of extracellular information by protein-tyrosine kinases and cell adhesion molecules. BioSystems, in press.

SEX DIFFERENCES IN THE DEVELOPMENT OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGF-R) SIGNALLING IN FETAL LUNG

Mo-20 H.C. Nielsen, D. Rosenblum, G. Yerzolimsky, M.V. Volpe, Y-S. Lo. Division of Newborn Medicine,
Department of Pediatrics, New England Medical Center,
Boston, Massachusetts, USA.

The maturation of the fetal lung is controlled by fibroblast-type II cell interactions. We and others have shown that these interactions occur earlier in female fetuses and are promoted by Epidermal Growth Factor (EGF). We proposed that the regulation of fetal lung maturation requires cell-specific development of EGF-R signalling. We studied EGF-R binding studies in primary cultures of fetal rat lung fibroblasts and type II cells prepared from gestations 17-22 of gestation. Female fibroblasts exhibited a 2-fold increase in EGF-R binding on day 18, and males exhibited a similar 2-fold increase on day 19. Type II cells exhibited only minimal binding which did not change with gestation. Western blot analysis similarly showed an increase in EGF-R protein in fibroblasts on day 18 in females and day 19 in males. Activation of the EGF-R was studied by measuring the production of diacylglycerol (DAG) in response to EGF. EGF first stimulated DAG production on day 18 in female fibroblasts by 2.5-fold, and on day 19 in male fibroblasts by 2.8-fold. No stimulation of DAG production by EGF was seen in type II cells. Immunohistochemistry was used to localize the EGF-R in vivo in the fetal lung. Immunoreactivity was observed along epithelial-epithelial and epithelial-mesenchymal contacts, and in clusters of mesenchyme underlying epithelium. We conclude that EGF-R expression in developing lung is an important regulatory mechanism in the process of cell-cell communication controlling fetal lung maturation.

Mo - 21

**EFFECTS OF MUTATED IP₃ RECEPTORS
ON SIGNAL TRANSDUCTION AND EGF-
DEPENDENT CELL GROWTH**

E. Clementi^a, G. Fischer^b, M. Raichman^a,
G. Racchetti^a, A. Ullrich^b, and J. Meldolesi^a.

^aChair of Pharmacol. Univ. of Reggio Calabria, Catanzaro; ^bDept. Pharmacol., Dibit-H San Raffaele Scientific Inst., Univ. of Milano, Italy; ^cMax Plank Institute für Biochemie, Martinsried, FRG.

The IP₃ receptor (IP₃R) is the omoctetrameric channel responsible for Ca²⁺ release from intracellular Ca²⁺ stores. The aim of this study was to reveal: i) the molecular mechanisms underlying the IP₃-induced signal transduction and ii) the role that IP₃ plays during cell growth and oncogenesis. To this purpose we generated stable NIH-3T3 transfectants expressing the human EGFR together with rat type I IP₃R subunits, wild type or deleted of the ligand binding domain (Δ form). The latter is known to be still assembled with wild type subunits to yield the tetrameric channel. In transfected clones both IP₃R synthesis and turnover were markedly increased. The mass accumulation was therefore only moderate (+15-30% of the endogenous IP₃R, for the Δ form). IP₃R containing the Δ form were found to be more sensitive to the ligand with respect to the controls. This change was accompanied by a decreased IP₃ generation at the ATP P_{2u} receptor, most likely due to a decrease in the expression of the P_{2u} receptor itself. The stimulated Ca²⁺ signalling was therefore affected only moderately in the transfected clones. In contrast, long term cell growth was significantly reduced (-60-70%) in the cells expressing the truncated receptor, while cell transformation induced by autocrine stimulation of the EGFR with TGF α was almost completely blocked (-90%). A negative correlation appears therefore to exist between IP₃R functioning and the growth properties of 3T3 cells.

Mo - 23

**HGFr-DERIVED ONCOGENIC ISOFORMS *tpr-met*
DIFFERENTIALLY REGULATE THE RESISTANCE
OF NIH-3T3 CELLS TO TNF α**

Y. Geffen^a, G.F. Vande Woude^b, S. Segal^a

^aDepartment of Immunology and Microbiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.
^bABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick Maryland 21702.

It was previously reported that the expression of the met protooncogene (HGFr) in NIH-3T3 cells is associated with increased resistance to the cytotoxic effects of TNF α . These findings suggest that a membrane receptor associated protein tyrosine kinase (PTK) may be involved in the regulation of signals exerted due to the ligation of TNF α receptors. Since TNF α is a potent regulatory entity of both basic and clinical oncological importance, experiments have been undertaken in our laboratory in order to: a) investigate whether indeed the HGFr associated PTK moiety is involved in conferring resistance to TNF α and b) whether the capacity of this moiety to influence the cellular response to TNF α is dependent on its molecular configuration. To investigate these questions we used NIH-3T3 cells (originally described by M. Park et al.), transformed by two different isoforms of the oncogene tpr-met which include the same PTK moiety of the HGFr but differ in the length of the amino terminal tpr moiety, both retain PTK activity. Our experiments have revealed that cells transformed by longer version of tpr-met are refractory to the cytotoxic effects of TNF α , while those transformed by the shorter version are susceptible to the cytotoxic effects of TNF α . Moreover, in serum free media the cells transformed by the longer isoform can grow rapidly in the presence of high concentrations of TNF α , unlike untransfected NIH-3T3 cells and cells transformed by the shorter isoform of tpr-met. Our data clearly indicate that the PTK HGFr associated moiety can confer resistance to TNF α and show that such protecting activity is possibly dependent on the exact cellular compartmentalisation of the PTK moiety and/or its susceptibility to the regulatory interactions with other molecules at the particular compartment.

Mo - 22

**DEPENDENCE OF EPIDERMAL GROWTH FACTOR
RECEPTOR ENDOSYTOSIS ON RECEPTOR
OCCUPANCY**

A.D Blagoveshchenskaya, I.P. Sokolova,
E.S. Kornilova, N.N. Nikolsky

Institute of Cytology, Russian Academy of Sciences, St.Petersburg

Two subpopulations of epidermal growth factor (EGF) receptor with different affinity to EGF have been demonstrated for many cell types. A key role of high-affinity receptors in stimulation of cell response to EGF has been suggested. In the present work an attempt has been made to study internalization and intracellular compartmentalization of high-affinity receptors. For this purpose endocytosis of ¹²⁵I-EGF in A431 cells was stimulated by low (less than 1 nM) EGF concentrations as well as after blocking of low-affinity binding sites with specific monoclonal antibodies 2E9. Subcellular fractionation in 17% Percoll gradient has shown that in both cases internalized ¹²⁵I-EGF was first found in light endosomes, then in heavy ones and remained there for a long time. The effectiveness of ¹²⁵I-EGF delivery to prelysosomal heavy endosomes as well as initial internalization rate is maximum at low EGF concentrations and is reduced dramatically with the increase of receptor occupancy. Monoclonal antibody Mab108 specifically recognizing high-affinity receptors is capable of stimulating receptor internalization, its initial rate being higher than that observed at high EGF concentrations. However, Mab 108-receptor complexes are localized in light endosomes only. Preincubation of cells at low concentrations of EGF resulted in redistribution of the bulk of ¹²⁵I-Mab 108 into heavy endosomes, which may be the result of tyrosine kinase activation of high-affinity receptor. Our data confirm the hypothesis of tyrosine kinase involvement in the regulation of both internalization and sorting of EGF-receptor complexes.

Mo - 24

**THE MITOGEN-ACTIVATED PROTEIN KINASES
SIGNALLING PATHWAYS IN VASCULAR SMOOTH
MUSCLE CELLS**

Yosef Granot, Hagit Fridman and Shirly Shaked. The Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva Israel

Mitogen-activated protein kinases (MAPK), are members of a 40-45 kDa family of serine/threonine protein kinases, that mediate the tyrosine kinase growth factor receptors signalling within cells. Our recent observations, provided direct evidence for MAPK activation by vasopressin (AVP) in vascular smooth muscle cells (VSMC) (Granot et al JBC 268, 9564, 1993). These observations, indicate the participation of MAPK activation in signalling pathways initiated by tyrosine and non-tyrosine kinase receptors.

A major question to be asked is: who are the common or the divergent functional proteins of these two signalling pathways? In the present studies we investigated the participation of the protein kinase C (PKC), and the MAPK kinase (MEK1) in the mediation of MAPK activation by AVP and EGF in VSMC.

The techniques used in these studies are: 1) Down regulation of PKC by chronic treatment of VSMC with PMA; 2) Identification of MAPK, PKC and MEK1 with specific antibodies; 3) Immunoprecipitation of the ³²P-radiolabeled proteins from AVP and EGF stimulated VSMC; 4) Determination of the MAPK activity in stimulated VSMC extracts; and 5) MEK function by using as a substrate wild type and kinase deficient purified MAPK produced in *E.Coli*.

Our observations demonstrate that: 1) PKC mediates the AVP but not the EGF MAPK activation; and 2) MEK1 phosphorylation and activation is not required for the AVP-dependent-PKC-mediated stimulation of MAPK.

These observations imply the existence of PKC mediated functional protein/s, in the MAPK activation signalling by neuropeptide hormones. This/e protein/s should be involved in the regulation of VSMC growth and proliferation by neuropeptide but not by tyrosine kinase hormone receptors.

Mo - 25 PASTEURELLA MULTOCIDA TOXIN, A MITOGEN FOR OSTEOBLASTS AND CHONDROCYTES

P.B. Mullan, and A.J. Lax

Institute for Animal Health, Compton, Newbury, Berkshire, RG16 0NN, United Kingdom

The *Pasteurella multocida* toxin (PMT) is a potent mitogen for Swiss 3T3 cells and stimulates DNA synthesis and cell growth in a manner equivalent to that induced by 10% serum. PMT is the main virulence factor in the pig disease atrophic rhinitis, which primarily affects formation of the nasal turbinete bones. We have investigated the effect of this toxin on osteoblast and chondrocyte cell cultures.

Primary chicken osteoblasts were liberated from embryonic calvariae by collagenase treatment, and a relatively pure population of alkaline phosphatase positive osteoblasts was obtained. These cells were quiesced. PMT induced a marked stimulation of DNA synthesis, and an increase in cell number in a dose dependant manner. There was no evidence of necrotic effects. Calvarial explants were used to produce a well-differentiated osteoblast population, which exhibited a similar increase in cell number in response to PMT.

We have also derived chondrocyte cultures from the proximal tibia of 3 week old chicks and found that PMT has a comparable stimulatory effect on cell proliferation.

These results indicate that PMT is a potent mitogen for cells involved in bone formation. PMT may prevent normal bone development by preventing differentiation.

SIGNALLING PATHWAYS IN SATELLITE CELL

Mo - 27 MYOGENESIS: RE-EXAMINATION OF THE ROLE OF PROTEIN KINASE C

I. Martelly^a, C. Lagord^a, M.P. Leibovitch^b, G. Carpentier^a, M. Castagna^c, J. Gautron^a et J. Moraczewski^d.

^a Lab Myogénèse et Régénération musculaire (MYREM-CRRET) CNRS URA 1813, Université Paris XII, Creteil, FRANCE; ^b CNRS URA 1158, IGR, Villejuif; ^c INSERM U268, Hôpital Paul Brousse, Villejuif, FRANCE; ^d Warsaw Univ. POLAND.

In regard to myogenesis, the signalling pathways through which environmental signals regulate this process are still unclear. Contradictory conclusions on the role of protein kinase C (PKC) in myogenesis appeared by using the PKC targeting drug, phorbol myristate acetate (PMA). In particular, PMA was shown to prevent fetal myoblast differentiation but not that of satellite cells isolated from adult skeletal muscles. This suggested several hypothesis: either PKC is not involved in the control of satellite cell myogenesis or PMA treatment has to be applied at an adequate moment in order to alter the myogenic process. Alternatively, PKC activity could be only partially down regulated in PMA-treated satellite cells. PKC activity is known, indeed, to belong to a family of proteins composed of several isoforms, including cPKC (α, β, γ) and nPKC (δ, ϵ, η) which can be differentially down regulated by sustained treatment with phorbol esters. In the present study we used primary cultures of rat satellite cells to reconsider the role of PKC in myogenesis.

We showed that -1) PKC activity changed during the myogenic process; -2) PKC activity was only partially down regulated by a sustained PMA treatment. -3) PMA treatment increased satellite cell differentiation when its application occurred at a specific moment of the cultures when satellite cells reached the post-mitotic stage. This effect correlated in time with changes in cAMP-dependent protein kinase (PKA) activity. 4) In an associated communication (Lagord et al) we showed that PKA inhibitors increase satellite cell myogenesis. PMA increased myogenesis by potentiated cAMP-dependent protein kinase inhibitor effects.

Taken together these results suggested that satellite cell PKC, which belongs probably to the nPKC isoform group, might stimulate myogenesis through an antagonistic effect on PKA.

PKA INHIBITORS ENHANCE MYOGENIC DIFFERENTIATION OF SATELLITE CELLS IN PRIMARY CULTURES AND IMPROVE IN VIVO MUSCLE REGENERATION.

C. Lagord^a, J. Moraczewski^b, J. Gautron^a and I. Martelly^a

^a Laboratoire Myogénèse et Régénération Musculaire (MYREM/CRRET), Université Paris XII-Val de Marne, Creteil 94010, France; ^b University of Warsaw, Poland.

Satellite cells are mononucleated myogenic cells located beneath the basal lamina of adult skeletal muscle. They are involved in the incorporation of new myonuclei during growth and repair of myofibers. In primary cultures, satellite cells proliferate and fuse into multinucleated myotubes. They undergo a coordinated expression of skeletal muscle-specific genes. Little is known about the intracellular signal routes which induce and regulate such processes. Especially, the role of signalling pathways involving cAMP-dependent protein kinase (PKA) is poorly understood.

Here, we show that treatments of satellite cells primary cultures with inhibitors of PKA activity (iso-H7 and HA 1004), increased biochemical and morphological differentiation, without altering cell proliferation. These effects are associated with an increased expression of myogenin but not of MyoD mRNAs. Furthermore, these drugs improve the *in vivo* regeneration of crushed rat muscle when injected in the muscle at the time of injury.

INHIBITION OF p-NITROPHENOL GLUCURONIDATION BY CA²⁺ MOBILIZING HORMONES

G. Banhegyi^a, L. Braun^a, R. Fulceri^b, A. Benedetti^b and J. Mandl^a

^a 1st Institute of Biochemistry, Semmelweis Medical University, Budapest, Hungary ^b Instituto di Patologia Generale, University of Siena, Siena, Italy

Glucuronidation is the most important pathway in phase II of biotransformation. The conjugation with UDP-glucuronic acid is catalyzed by UDP-glucuronosyltransferases. UDPGT isoenzymes are integral membrane proteins of the endoplasmic reticulum; their active site is in the luminal compartment of the ER. Previously we have shown that decrease of intraluminal Ca²⁺ concentration by Ca²⁺-ATP-ase inhibitors resulted in a lower activity of UDPGT. In the present paper the effect of two receptor mediated Ca²⁺ mobilizing agent - vasopressin and phenylephrine - on the glucuronidation of p-nitrophenol was investigated in isolated mouse hepatocytes. Vasopressin and phenylephrine significantly inhibited the glucuronidation of p-nitrophenol. Depletion of the intracellular Ca²⁺ stores by preincubating the isolated hepatocytes in the presence of excess EGTA itself caused a 70% decrease of p-nitrophenol conjugation. Under these circumstances the inhibition became unsusceptible to the addition of the hormones. Consequently, the inhibitory effect depended on the Ca²⁺ filled state of the intracellular Ca²⁺ stores. The inhibition caused by α_1 -agonist phenylephrine was reversible and could be suspended by the addition of α_1 -antagonist prazosin. These results suggest that the intracellular Ca²⁺ mobilization plays dominant role in the short-term regulation of UDPGT activity, therefore, may influence the elimination of endo- and xenobiotics.

EXPRESSION AND CHARACTERISATION OF A NOVEL SRC HOMOLOGY 2 PROTEIN IN β TC-1 CELLS

Mo-29 J.Mares^a, C.Lavergne^b, B.Breant^b, M.Welsh^c
^aInstitute of Biology and Medical Genetics, Charles University, Prague, Czech Republic, ^bINSERM, U.55, Hopital Saint-Antoine, Paris, France, ^cDepartment of Medical Cell Biology, Uppsala University, Sweden

To characterize serum-inducible genes in the insulin-producing β TC-1 cells, a library subtraction screening procedure was performed on serum-deprived (G_0) and serum restimulated (G_1) β TC-1 cells. A cDNA containing a motif with strong homology to src homology 2 (SH2) domains was found using this procedure and called Shb. The cDNA hybridized to a similar or identical mRNA of 3.1 kb expressed in mouse brain, liver, kidney, heart, NIH3T3 fibroblasts and β TC-1 cells. A fusion protein of Shb SH2 domain and glutathione S-transferase showed increased binding to glycoproteins of the cells stimulated with platelet-derived growth factor (PDGF-BB).

It is concluded that Shb is a novel SH2-containing protein with possible SH3-binding ability involved in the signal-transduction of ligand-activated tyrosine kinase receptors.

GENERATION OF DIFFERENTIAL CELL LINES CONTROLLED BY THE VIMENTIN PROMOTER

Mo-31 P.Vicart, B.Schwartz, C.Babinet and D.Paulin
 Biologie moléculaire de la différenciation - Institut Pasteur - Paris France

Differentiated clonal cell lines were obtained from human tissues and transgenic mice carrying a recombinant gene composed of DNA coding for a temperature-sensitive mutant of the simian virus large T antigen under the control of regulatory elements of the human vimentin gene. In response to mitogenic factors the vimentin promoter is activated in the presence of serum in all cultured cells independently of their origin. The expression of the T antigen could be controlled both at the level of transcription since the vimentin promoter is growth-regulated and at the level of the protein structure through the temperature stability of the T antigen. Indeed, the switch off of the oncogene protein is obtained by serum deprivation of the culture and achieved with enhancement of the growth temperature. From transgenic mice a large variety of clonal differentiated cell lines were established and characterized including melanocytes, macrophages, mesangial, muscle and endothelial cells. Melanocytes displayed melanin while endothelial cells from brain and heart expressed the related factor VIII and low density lipoprotein absorption capacities. Mesangial cells from kidney exhibited numerous desmosomes. Typical markers of macrophages from bone-marrow were observed while skeletal muscle cells fused and contracted.

INDUCTION OF OLIGODENDROCYTE DIFFERENTIATION IN RSV-TRANSFORMED CHICK NEURAL CELLS

Mo-30 M.Lišková, V.Sovová, M.Wimmerová, D.Trnka, E.Šloncová and H.Fidlerová
 Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

A novel chicken cell line (NRCH68) was established from a mixed tissue culture of chick embryo neural retina and chorioidea cells transformed by ts-RSV (NY-68). NRCH68 cells produced ts-RSV; pp60 γ -SRC kinase activity was detected in these cells at the permissive (35-37°C) temperature. No pp60 γ -SRC kinase activity was detected at the non-permissive (39.5-41°C) temperature. In contrast to murine cells in which the expression of v-src was reported to arrest glial cell differentiation, NRCH68 cells were induced by bromodeoxyuridine (BrdU) to differentiate to oligodendrocyte (O4-positive) cell type at the permissive temperature only. No O4-positive cells were found in BrdU-treated NRCH68 cells at the non-permissive temperature. Results suggest that the NRCH68 cell line is a promising model for investigating the interrelations between cell differentiation and oncogenic transformation.

STIMULATION OF CELL GROWTH BY HEAVY METALS

Mo-32 T.von Zglinickia and J.Wroblewskib
^aInstitut für Pathologie, Charité, Humboldt-Universität Berlin, Germany; ^bDept. of Medical Cell Genetics, Karolinska Institute, Stockholm, Sweden

Contradictory reports concerning the growth stimulatory potential of cadmium (Cd) and mercury (Hg) prompted us to study the heavy metal-dependent cell proliferation and the possible mechanisms involved. While micromolar concentrations of Cd and Hg are inhibitory, both metals in nanomolar or lower doses stimulate proliferation of different cell lines including LLC-PK₁ renal epithelial cells and L6J1 myoblasts. Cd in low concentrations also stimulates the accumulation of *c-myc* and *c-jun* proto-oncogene mRNA, but neither *c-fos* nor *metallothionein*. These effects are not mediated by changes in intracellular free calcium or pH, but are accompanied by changes in intracellular K and Cl concentrations as measured by X-ray microanalysis. It is concluded that shifts in nuclear monovalent ion concentrations might be of regulatory importance for oncogene activation and the stimulation of cell proliferation by heavy metals.

A NEW PRINCIPLE OF CLASSIFICATION
OF TUMOURS

Mo-33 G.V.Vikha, R.G. Vasilov

Research Institute of Biotechnology,
Moscow, Russia

Intensity and distribution immunohistochemical staining of tumour cells depend on the localization and the feature forming of tumour structure. So there is actual task to create the database about the results immunological reaction of the different reagents with the structure components of the tumor cells. On the base of these dates it is possible to create immunological atlas of tumors and to develop the classification characteristics of tumors. It is important that the characterizations of tumor cells and tumor slice have been studied under a standard set of conditions. The first type of characteristics of tumor is a marker of tumor cells. In this part it is necessary to involve composition, structure, square of the cell surface occupied by marker and a percentage of marker expression. The second type of characterizations is photograph of slice of tumor tissue after immunofluorescent and immunoenzyme staining by one or two stage technique. The main characterizations are intensity and distribution of label (FITC or enzyme), the type of localization (membrane, cytoplasm etc.). The important condition of creation successfully working rule of classification is the choice of normal control example. Total structure of immunological atlas will be presented.

STRUCTURAL AND ULTRASTRUCTURAL LOCALIZATION
OF THE V-MYB AND C-MYB GENE PRODUCTS IN CELLS
OF THE BACULOVIRUS EXPRESSION SYSTEM

Mo-34

J. Štokrová^a, J. Korb^a, M. Dvořáková^a,
V. Čermáková^a and I. Raška^b

^aInstitute of Molecular Genetics CZ AS, Prague, Czech Republic; ^bInstitute of Experimental Medicine CZ AS, Prague, Czech Republic

We used the baculovirus expression system for studying the localization of v-myb oncogene and c-myb protooncogene products on cellular structures. All analyses were performed on *Spodoptera frugiperda* SF9 and SF900 cells, infected with recombinant baculovirus containing v-myb or c-myb gene inserts. As a control system the uninfected cells or cells infected with wild type of baculovirus served. For a precise localization of v-myb and c-myb gene products by means of both immunofluorescent and immunoelectron microscopy specific polyclonal antibodies against v-myb protein were prepared. The monoclonal antibodies against fibrillarin or DNA were used as a characteristic marker of nucleolar components and nuclear chromatin structures, respectively.

We found that the v-myb oncogene product was localized mostly in the central area of the cell nucleus, but some amount of this protein was also detected in distinct domains residing in the nuclear membrane area. There was clear coincidence of this product with DNA marker, whereas its coincidence with fibrillarin, a typical nucleolar marker, was controversial. On the other hand, the c-myb protooncogene product decorated mostly the ring-shaped central area of nucleus, but was seldom detected in the nucleus periphery.

This different distribution of v-myb and c-myb gene products might reflect the differences in their functional properties.

MORPHOLOGICAL CHANGES OF THE INSECT CELLS IN
THE BACULOVIRUS EXPRESSION SYSTEM AS A
FUNCTION OF V-MYB AND C-MYB GENE INSERTS

J. Korb, J. Štokrová, M. Dvořáková, V. Čermáková and V. Karafiát

Institute of Molecular Genetics CZ AS, Prague, Czech Republic

The morphology of the insect cells *Spodoptera frugiperda* of the baculovirus expression system containing v-myb oncogene and c-myb protooncogene inserts were analysed by means of both optical and electron microscopy. This morphology was compared with those of the cells of the baculovirus system lacking these inserts.

We have shown that expression of c-myb and especially v-myb genes was accompanied by extensive structural changes of the cell nucleus and its components, which was apparent as quickly as 48 hours after infection. Ultrastructural analyses revealed an increase of nuclear content which, in the case of v-myb gene expression, was enormous and was accompanied by changes in the cytoplasm which practically disappeared. Typical baculovirus particles were observed only in the nuclear area of the infected cells. Nucleolus characteristic of the intact cells was apparently disintegrated, due to v-myb and c-myb gene expression.

The observed structural changes of cell nucleus are probably due to strong interactions of v-myb and c-myb gene products with nucleolar and nuclear components.

THE EFFECT OF BUTYRIC ACID AND ITS PRODRUG
ON THE EXPRESSION OF SUPPRESSOR GENES IN
LEUKEMIC CELL LINES

Mo-36

E. Rabizadeh^a, M. Shaklai^a and A. Rephaeli^{ab}

^aFelsenstein Medical Research Center and Hematology Division, Beilinson Medical Center, Petah Tikva 49100 & Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; ^bAnsan Inc., 400 Oyster Point Blvd. Suite 315 S. San Francisco, Ca, 94080 U.S.A.

Butyric acid (BA) was shown to modulate the expression of oncogenes and suppressor genes in several cell types. The novel derivative of BA, pivaloyloxymethyl butyrate (AN-9), affects the cells and modulates the expression of the oncogenes c-myc and c-jun, at lower concentrations and faster than BA. We have examined the effect of BA and AN-9 on the expression of suppressor genes Rb and p53, in the promyelocytic human cell line (HL-60) and murine erythroleukemia cell line (MEL) respectively. The expression of Rb in HL-60 cells increased by over 4-fold after 24h of exposure to BA (0.5mM) and AN-9 (0.075mM). In the MEL cells, BA at 1mM augmented the level of p53 by 35%, after 3h of exposure, while at the same time AN-9 at 0.075mM and 0.25mM augmented the expression of the gene by 25% and 80% respectively. Previously we have shown that AN-9 decreased the expression of c-myc. Staurosporine, a protein kinase C (PK-C) inhibitor, abrogated the inhibitory effect of AN-9 on c-myc and diminished the stimulatory effect of AN-9 on Rb expression. Taken together, the above observation supports the involvement of PK-C in the differentiation pathway induced by AN-9.

Mo - 37 SPATIAL TARGETING OF C-MYC mRNA TO DIFFERENT INTRACELLULAR SITES OF TRANSLATION: ROLE OF 3' UNTRANSLATED REGION AND CYTOSKELETON

J.E. Hesketh*, G.P. Campbell*, N. Loveridge* and J. M. Blanchard^b

*Rowett Research Institute, Bucksburn, ABERDEEN, AB2 9SB, UK
and ^bInstitut de Génétique Moléculaire, URA CNRS 1191, 34095 MONTPELLIER, France

Targeting of proteins to their site of function plays a major role in cell organization. This is partly achieved by signals within the polypeptide chains but recent data suggest that there is also targeting of mRNAs to different intracellular sites. mRNA localization may involve interaction of mRNAs with the cytoskeleton and has been suggested to involve sequences in the 3' untranslated region (3'UTR) of the mRNAs. The influence of the 3' UTR on mRNA localization was investigated by measuring the distribution of myc, β -globin and hybrid myc-globin mRNAs between free, cytoskeletal-bound and membrane-bound polyosomes in cells transfected with either control or chimaeric gene constructs. C-myc sequences and β -globin coding sequences linked to the myc 3' UTR were present at greatest enrichment in cytoskeletal-bound polyosomes. β -globin mRNA and myc coding sequences linked to the β -globin 3' UTR were recovered largely in the free polyosomes. In situ hybridization showed that replacement of the c-myc 3'UTR by that of globin caused a relocalisation of the mRNA from perinuclear to peripheral cytoplasm. Immunocytochemical detection of c-myc protein showed that this was accompanied by altered protein distribution, more being present in the peripheral cytoplasm and proportionally less in the nucleus. The results indicate that mRNA localization in mammalian cells involves directional information in the 3' UTR of mRNAs. Furthermore correct mRNA localization is not obligatory for translation to occur, although it appears that incorrect localization reduces the efficiency of subsequent protein targeting.

Mo - 39

SEARCHING FOR THE FUNCTION(S) OF THE p53 SUPPRESSOR GENE IN NORMAL CELLS

Varda Rotter, Ronit Grinstein-Aloni, Dov Schwartz, Barry Elkind, Arnold Simons, Roland Wolkowicz, Pierre Beserman, Ahuva Kapon and Naomi Goldfinger. Department of Cell Biology, The Weizmann Institute, Rehovot, ISRAEL

Inactivation of the p53 tumor suppressor gene plays a major role in malignant transformation. Our *in vitro* experiments have suggested that p53 is involved in cell differentiation in the B-cell lineage. When constitutive expression of wild-type p53 was instituted in a pre-B p53 nonproducer cell line, the cells advanced in their stage of differentiation. Under *in vivo* conditions, expression of wild-type p53 in those p53-nonproducer pre-B cells, caused them to develop smaller size tumors at a lower frequency than following injection of the parental p53 nonproducers. The conclusion that p53 plays a role in normal development and differentiation *in vivo* is substantiated by our studies with p53-promoter-CAT hybrid transgenic mice. We found that p53 expression in adult mice is significantly high in the testes, where it might play a role in the process of sperm cell differentiation and maturation. Analysis of DNA content coupled with p53 expression showed that p53 is expressed in a temporally regulated fashion during spermatogenesis and is confined to the pachytene stage of the meiotic cell cycle. We found that the endogenous levels of p53 mRNA and protein of the p53-promoter CAT transgenic mice were reduced compared to the non-transgenic control mice. The various p53-promoter-CAT transgenic mice exhibited in their testes multinucleated giant cells, a degenerative syndrome resulting from the inability of the tetraploid primary spermatocytes to complete meiotic division. The giant cell degenerative syndrome was also observed in some genetic strains of p53 null homozygotic mice. In view of the hypothesis that p53 plays a role in DNA repair mechanisms, it is tempting to speculate that the physiological function of p53 that is specifically expressed in the meiotic pachytene phase of spermatogenesis is to allow adequate time for the DNA reshuffling and repair events which occur at this phase to be properly completed. Primary spermatocytes which have reduced p53 levels are probably impaired with respect to their DNA repair, thus leading to the development of genetically defective giant cells that do not reach maturation.

EXPRESSION PATTERN OF DEVELOPMENTAL REGULATORS IN HUMAN PLACENTA AND EMBRYOFETAL TISSUES

Mo - 38

D. Rossi*, L. Del Giacco*, L. Doneda*, B. Acaia^a, D. Brioschi^a, U. Nicolini^a, L. Larizza*

*Dept. of Biology & Genetics ^a I Dept. of Obstetrics & Gynaecology University of Milan, Italy

The expression pattern of EGFR, c-sis, c-fos and c-jun was studied in developing organs from first trimester aborted human foetuses and placenta specimens spanning the whole gestation. Embryofetal organs analysed were brain, gut, liver, kidneys, lungs, heart from the 8th-13th week of gestation, the 10th-13th week being the most widely represented period. Northern analysis of all the protooncogenes under study in the above organs pointed to the brain as the organ with the widest spectrum of transcriptional activity. Indeed EGFR, c-sis, c-jun specific transcripts were detected in the brain starting from the 10/11th week of gestation. EGFR was also found to be expressed in the liver from the 8th week and c-sis in the gut from the 12th week. No other positivities were recorded. RT-PCR analysis of c-fos in brain, gut, liver, kidney, lung and heart from 13-week embryos showed expression of the gene in all organs. Time-course analysis evidenced expression of c-fos in brain and gut from the 10th to the 13th week. Northern analysis of 33 placenta specimens, ranging from the 5th to the 39th week of gestation showed EGFR-specific transcripts in all samples, with more consistent levels in the third trimester. No appreciable levels of c-jun specific mRNAs were detected in any sample. C-fos specific transcripts were not apparent till the 19th week of gestation, but were clearly detected from that period to the end. C-sis expression was also found to be modulated, being detectable only from the 8th week of gestation. RT-PCR analysis allowed c-fos transcripts to be detected in all the placenta specimens from the 5th to the 39th week of gestation.

Supported by AIRC.

Mo - 40 NON-ISOTOPIC DETECTION OF SINGLE STRAND CONFORMATION POLYMORPHISM (PCR -SSCP) AS A TECHNIQUE FOR SCREENING OF MUTATIONS IN NF 1 GENE

M. Korápečná^a, V. Matoška^b, I. Mazura^b, A. Petráková^b, J. Reischig^a and E. Žďárský^b

^aDepartment of Biology, Medical Faculty of Charles University, Pilzen, Czech Republic,

^bDepartment of Molecular Biology, 3rd Medical Faculty of Charles University, Prague, Czech Republic.

Von Recklinghausen neurofibromatosis, type I (NF1), is one of the most common hereditary disorders in humans (incidence 1:3000). Several recent studies have demonstrated that the NF 1 gene may not only be mutant in typical NF 1 tumours but also in a variety of seemingly unrelated neoplasms. The product of this gene called neurofibromin belongs to the GTP-ase activating proteins, and plays an important role in cell cycle regulation and cell differentiation. At present, the "hot spots" for mutations within the NF 1 gene are very intensively searched for.

In order to select the convenient fragments of NF1 gene for a sequence analysis, we have established a PCR-SSCP method utilising the Bio-Rad Silver Stain Kit. In this paper we present our experience as well as the results obtained by this method.

Mo-41 FREQUENCY AND SPECTRUM OF THE K-RAS MUTATIONS IN HISTOLOGICAL SUBTYPES OF NON-SMALL-CELL LUNG CANCER

J. Vachterheim, I. Horáková, H. Novotná

Laboratory of Molecular Biology, Institute of Chest Diseases, Prague, Czech Republic

Lung cancer is one of the leading causes of cancer death approaching 6000 cases in the Czech Republic in 1993. Mutations in *K-ras* oncogene have been reported to occur and suggested to play a role in lung adenocarcinomas. We examined 141 lung tumor DNA samples for mutations in codons 12, 13, and 61 of the *K-ras* oncogene. For the codon 12, we used sensitive two-step PCR-RFLP method which detects as low as 1 per cent of mutated DNA in the sample. The types of mutations were determined by direct sequencing of ds PCR products. Total number of 19 mutational events were found (15 in codon 12, 3 in codon 13, and 1 in codon 61). The frequency was 8 of 20 adenocarcinomas (40%), 10 of 117 squamous cell carcinomas (8%), and 1 of 4 large-cell carcinomas. Most mutations were G to T transversions and G to A transitions. In codon 12, the point mutations resulted in the following amino acid changes: from glycine (wild type) to cysteine (4 samples), to aspartic acid (4 samples), to valine (2 samples), to serine (2 samples), to alanine (1 sample) and to phenylalanine (two nucleotide changes, one sample). Similar mutational spectrum was found in both squamous cell carcinomas and adenocarcinomas, suggesting similar carcinogenic pathways in both histological types of the tumor. We conclude that *K-ras* mutations can play a role in the development of not only lung adenocarcinomas but also of a subset (about 8 per cent) of squamous cell carcinomas.

Mo-43 THE MOUSE HTF9-A / RAN-BP1 GENE IS REGULATED IN THE CELL CYCLE BY THE E2F FACTOR AND THE RETINOBLASTOMA GENE PRODUCT

P. Lavia, G. Di Matteo, P. Fuschi, M. Salerno, R. Ricordy, Centro di Genetica Evoluzionistica del CNR, c/o Università "La Sapienza", Roma 00185, Italy.

We have isolated the mouse gene *Htf9-a* by virtue of its association with a CG-rich promoter. Recently, the gene was independently shown to encode the Ran-binding protein, which is associated with the nuclear GTP-binding Ran protein: ran-BP1 selectively interacts with the GTP bound, i.e. active, form of Ran. The latter is implicated in various cellular processes, such as DNA replication, RNA and protein import, and cooperates with the mitotic regulator RCC-1 in controlling the onset of mitosis after completion of DNA synthesis.

We found that expression of the *Htf9-a* / RanBP1 gene, though ubiquitous among cell types, was dependent on the proliferating ability of the cells. An E2F-binding site in the promoter proved to be required for cell-cycle regulation of the gene. The E2F factor, or factors of similar DNA-binding ability, bind to promoter elements common to many cell cycle genes. The trans-activating ability of E2F is abolished by the association with the p105 product of the retinoblastoma gene (RB): p105 can be thought of as a transcriptional repressor, and displays its antioncogenic activity in the G0/G1 phase by inhibiting the activity of E2F on several cell cycle genes. Expression of the E1A oncoprotein, which inactivates p105/RB, prevented down-regulation of *Htf9-a* in resting cells; this implicates the RB gene in negative control of *Htf9-a*.

Together these observations indicate that the *Htf9-a* gene is a novel regulatory target gene of E2F in cycling cells while being under the negative control of p105 in quiescent cells; the functional antagonism between these two regulatory molecules may provide the basis for the coordination between *Htf9-a* activity and the cell cycle.

Mo-42 CELL CYCLE ALTERATIONS AND p53 EXPRESSION IN TUMOR CELLS AFTER DNA DAMAGE

R. Supino^a, F. Napolitano^a, G. Bottiroli^b, E. Prosperini

^aIstituto Nazionale Tumori, ^bMilan, Italy; Centro di Studio per l'Istochimica del CNR, Pavia, Italy

Wild-type p53 is involved in the cellular response to DNA damage. The modulation of p53 expression after DNA damage induced by γ -rays or etoposide was investigated with immunocytometry in a human small cell lung carcinoma (SCLC) cell line. A different kinetics of p53 expression after the two treatments was found. A moderate increase in p53 was observed 1 h after irradiation (8 Gy), but already at 2 h it decreased to the control values. At 24 h, when cells were arrested in the G₂ phase of the cell cycle, another increase was observed. In contrast, cells treated with etoposide showed an increase in p53, starting 14 h after treatment, that was maintained until 48 h. Concomitantly with these changes, cells were arrested in the G₂ phase of the cell cycle. The increase in p53 induced by etoposide, or by γ -rays at 24 h after irradiation, is likely to be related to the arrest of cells in G₂, given that cells in this phase have higher p53 levels than those found in G₁. On the contrary, the p53 increase observed 1 h after irradiation appears to be due to an accumulation of the protein since, at this time, no alteration in the cell cycle was evident. The modulation in p53 expression in this cell line may be related to the low levels of the wild-type protein and the relatively high levels of the mutated form (found in SCLC) that is not involved in the cellular response to DNA damage.

Mo-44 ROLE OF PROTEIN KINASE C IN RAS-MEDIATED FOS EXPRESSION.

Florian Überall, Sonja Kampfer, Christian Schubert, Wolfgang Doppler and Hans H. Grunicke, Institute of Medical Chemistry and Biochemistry, Fritz Preglstrasse 3, A-6020 Innsbruck, AUSTRIA. The mechanism by which transforming Ha-ras induces c-fos expression in HC11 mouse mammary epithelial cells was investigated with regard to controversial data concerning the role of protein kinase C (PKC) and the required promoter elements of the fos gene. HC11 cells carrying a glucocorticoid-inducible Ha-ras (val12) construct were transfected with a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a human fos promoter which includes the serum response element (SRE), the adjacent c-fos AP-1 site (FAP) and the cAMP response element (CRE). Induction of the Ha-ras gene by dexamethasone lead to a transactivation of expression of the transfected fos promoter construct which was inhibited by the PKC inhibitor BM41440 and abrogated in PKC-depleted cells. A similar transactivation was observed when the fos promoter construct was cotransfected with a constitutively active ras expression vector. Again, this effect was depressed by the PKC inhibitor and abolished in PKC-depleted cells. "PKC-depletion" was achieved by long-term exposure to TPA. This procedure was shown to deplete cells of PKC α and to significantly reduce PKC ϵ . Long-term exposure to bryostatin 1 selectively depletes PKC α . Depletion of PKC α by bryostatin 1 does not reduce the transcriptional activation of the SRE-FAP-TK-CAT construct by Ha-ras. In order to delineate the promoter elements mediating the transcriptional activation, constructs which lack the FAP and the CRE sites but contain an intact SRE were cotransfected with the ras construct. Elimination of the FAP and CRE sequences did not affect the transcriptional activation by Ha-ras (val12). It is concluded that in HC11 cells, transforming Ha-ras activates c-fos expression in a PKC-dependent manner presumably implying PKC ϵ and that the SRE is sufficient to mediate transcriptional activation.

MO - 45 INTERLEUKIN-1 (IL-1) ACTIVATION OF HYDROGEN PEROXIDE PRODUCTION BY LIVER SINUSOIDAL ENDOTHELIAL CELLS INDUCES THE IN VITRO LYSIS OF B16 MELANOMA CELLS.

G. Avivi, J. Anasagasti, and F. Vidal-Vanaclocha.

Department of Cell Biology & Morphol. Sciences, Basque Country University School of Medicine, Leioa, Vizcaya 48940, Spain.

Despite the importance of initial tumor-endothelial cell interactions in the selection of metastatic sites, intracapillary survival of tumor cells is an additional requirement in the metastatic progression. To analyze this post-adhesion event, we have developed an *in vitro* adhesion assay between sinusoidal endothelial cells, isolated from the liver of syngeneic mice, and a liver-metastasizing variant of B16 melanoma. In this assay, the time courses of hydrogen peroxide production by endothelial cells and tumor cell lysis were simultaneously determined. Production of hydrogen peroxide was quantified by multiwell plate-scanner fluorimetry of intracellular 2',7'-dichlorofluorescein (DCFH) oxidation by endothelial cells. Tumor cell viability was revealed on the basis of ethidium homodimer (EH) binding to cell nucleus and carboxyfluorescein (BCECF) leakage from the cytoplasm, both detected in damaged cells by the same fluorimetric device.

Since the first hour of B16 melanoma cell adhesion, a progressive increment of hydrogen peroxide production by targeted endothelial cells was detected. This response was in correlation to the number of added tumor cells, until a plateau at a tumor-endothelial cell ratio of 1:1. The maximum response occurred at the 2nd-to-3rd hour interval of the co-culture, being three times higher than in unstimulated endothelium. Hydrogen peroxide production by human recombinant IL-1 β treated endothelial cells showed a dose- and time-dependent response to this cytokine (maximum at 1ng/ml and on the 6th hour). The produced hydrogen peroxide was quickly released to the extracellular medium. In fact, tumor cell incubation in the presence of conditioned medium from 24h cultured endothelial cells significantly ($p<0.01$) increased EH binding to cell nucleus and reduced cytoplasmic BCECF. This tumor cell damage significantly increased when endothelial cells were pre-incubated with IL-1, being significant the correlation ($r=0.9$) between hydrogen peroxide production and tumor cell lysis. Endothelial cells from mouse liver sinusoids responded to B16 melanoma cell adhesion through a rapid production of hydrogen peroxide which damaged tumor cells. This effect was up-regulated by IL-1, a cytokine produced by melanoma cells and sinusoidal endothelium.

ROLE OF THE RAS GENE PRODUCTS IN SACCHAROMYCES CEREVISIAE MORPHOGENESIS AND LIFE SPAN

MO - 47

D. Vondráková^a, A. Pichová^{a,b}, E. Streiblová^a and M. Breitenbach^b

^aLaboratory of Cell Reproduction, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic; ^bInstitute of Genetic, University of Salzburg, Salzburg, Austria

Two ras genes designated RAS1 and RAS2 have been identified in the genome of *S.cerevisiae* (Powers et al., *Cell* 36, 607). Current evidence indicates that their products play an important role in the spatial control of morphogenesis and aging in this yeast. Budding yeasts offer an unique opportunity to study both phenomena using fluorescence microscopy. Following each cell division, a bud scar is formed at the cell surface of the mother cell at the site of separation of the daughter cell. Thus, the number of bud scars indicates the number of times the cell has divided and provides a metric system to monitor the nominal age and life span of individual cells. The topology of bud scars provides a record of spatial cell cycle control and the cytoskeleton plays a key role in the localization of the budding sites.

To understand the role of RAS gene products, wild type and mutant cells were examined by fluorescence microscopy of Calcofluor-, antitubulin- and F-actin staining at the level of individual cells and cell populations. Age distribution of RAS2⁺ and RAS2^{val19} populations will be presented as compared with RAS2⁺.

MO - 46 IMMUNOHISTOCHEMICAL ANALYSIS OF PHOSPHOTYROSINE, p62c-myc AND p21Ha-ras IN NORMAL AND NEOPLASTIC MURINE COLONOCYTES

B. Schwartz^a, D. Benharoch^b, I. Prinsloo^b, E. Cagnano^b, Y. Niv^a, S.A. Lamprecht^a, ^aGastroenterology Department, Clinical Biochemistry Unit and ^bInstitute of Pathology, Soroka Medical Center, Ben-Gurion University of the Negev, Beer-Sheba, Israel.

In the present study we used monoclonal antibodies to investigate the expression of phosphotyrosine, c-myc and Ha-ras proteins along the crypt continuum of normal and transformed rat colon tissue. Colon cancer was induced by administration of dimethylhydrazine (DMH). The immunohistochemical analysis of tyrosine-phosphorylated proteins in the normal rat indicate that positive staining was restricted to the lower colonic crypt zones. The carcinogenic insult altered the magnitude and positional profile of phosphotyrosine along the colon crypt axis during the preneoplastic period. The strongest staining was evident in invasive adenocarcinoma tissue. In contrast to phosphotyrosine, the feeble c-myc immunohistochemical staining of normal rat colonic crypts did not exhibit a preferential topology. However, following DMH administration and prior to frank neoplasia a substantial increase in the staining intensity for c-myc was noted, confined mostly to the supranuclear region of luminal cells. Invasive adenocarcinoma tissue displayed intense cytoplasmic c-myc immunoreactivity. The p21 Ha-ras protein was markedly expressed in colonic surface epithelium of normal and DMH-treated rats. Proliferating colonic cells resident in the lower crypt regions were negative, whereas midcrypt colonocytes exhibited moderate p21 ras staining.

The present immunohistochemical observations indicate that chemically-transformed murine colonic epithelial cells exhibit early, premalignant changes in tyrosine phosphorylation and in c-myc expression associated with an abnormal positional profile of the oncoproteins along the crypt axis. The carcinogenic insult did not alter the expression and topology of the Ha-ras gene product.

ANTI-TUMOR EFFECT OF IMMOBILIZED AND MEMBRANE-BOUND FORMS OF TUMOR NECROSIS FACTOR

Domonkos Á., Nagy, T., Degling, L., Anghel, C., Duda, E.

MTA Biol. Res. Center, Szeged, Hungary and Medical Products Agency, Uppsala, Sweden

We have found in previous experiments that a number of different tumor cell lines that synthesized significant amounts of human tumor necrosis factor (hTNF) had lower tumorigenicity *in vivo* than their parental cell lines. Mice inoculated s.c. at different locations with the TNF producing and the parental tumor cells frequently rejected both tumors, though circulating TNF levels were usually low or non-detectable. All the used cell lines and the parental lines were insensitive to TNF *in vitro*.

Tumors producing the transmembrane form of TNF (26 kDa preTNF) also induced the rejection of tumors arising from simultaneously injected parental cells, too. This observation suggested that TNF production induced tumor rejection by mechanism(s) other than direct cytotoxicity or vascular endothelial activation. We assumed that high local levels of TNF upregulated antigen presentation of the tumor cells and activated cytotoxic immune cells.

Since circulating TNF has high systemic toxicity and the transmembrane form of TNF is able to elicit TNF specific signalling in target cells, we produced different forms of membrane bound and immobilized TNF derivatives. Some of these preparations exhibited very effective anti-tumor properties *in vitro* and *in vivo* without serious side effects.

STUDY ON THE ASSOCIATION OF T ANTIGEN WITH
HUMAN BRAIN TUMORS.

Mo - 49

P.Carinci^a, M.De Mattei^a, F.Martini^a, M.Gerosa^b,
A.Corallini^c, G.Barbanti-Brodano^c, M.Tognon^a.

^aInst.Histology Gen.Embryol. and ^cMicrobiology, University of FERRARA, 44100 FERRARA; Dept.of Neurosurgery, University of VERONA, 37100 VERONA, Italy.

In our investigation 13 human normal brain tissues, 48 primary brain tumors and 10 cell lines derived from them were analyzed for DNA and RNA sequences homologous to the T antigen of polyomaviruses. PCR amplification using specific primers and hybridization with internal oligoprobes allowed to detect the sequences of the human polyomavirus BK. Moreover, 25 of 35 samples of peripherical blood cells from healthy donors, used as a control, were also positive. DNA sequencing data obtained from 5 brain tumors and 1 normal brain confirmed that the amplified sequences belong to the early region of BKV-DNA. Expression of this region was detected by RT-PCR in 5 human tumor cell lines. Our results confirm BKV latency in normal brain and peripheral blood cells. In addition, we found that the large T antigen is associated with different brain tumors. This phosphoprotein of 90K, which is located in the nucleus of the cells, induces extensive chromosomal rearrangements and binds the products of tumor suppressor genes p53 and RB, inactivating their functions.

DETERMINANTS OF THE BINDING SPECIFICITY
AND POLARITY OF RXR, RAR AND TR FULL-

Mo - 50

LENGTH RECEPTORS TO DIRECT REPEATS
C.Zechel, X.-Q. Shen, J.-Y. Chen, P. Chambon
and H. Gronemeyer.

INSERM-U.184/CNRS-LGME, Institut de Chimie Biologique, Faculté de Médecine, 11, rue Humann, F-67085 Strasbourg, France

Selective recognition of cognate cis-acting DNA elements by trans-acting regulatory proteins is crucial in the cascade of events that ultimately leads to the realization of specific genetic programs, which in the case of the nuclear receptors control multiple aspects of development, cell growth and differentiation, and homeostasis. We found that the DNA binding domains (DBDs) of RAR, RXR and TR dictate the response element recognition of the corresponding full-length receptors by steric hindrance phenomena, and by homo- and heterocooperative interactions. The dimerization surfaces located in the DBDs of RXR, RAR and TR, which are responsible for the cooperative interactions on directly repeated PuG(G/T)TCA motifs spaced by 1-5 bp were defined. Dimerization interfaces formed between the C-terminal RXR zinc finger (CII) and the T-box of either RAR or RXR are responsible for the cooperative binding to DR2 (RXR and RAR) and DR1 (RXR and RXR), respectively. The D-box of the RXR CII finger and the tip of the N-terminal RAR zinc finger (CI) are specifically required for heterodimerization on DR5, while the RXR D-box and a 7 amino acid sequence immediately N-terminal to the TR CI are specifically required for efficient RXR-TR dimerization on DR4. Modelling of these various interactions and crosslinking revealed that the RXR DBD providing the CII surface has to occupy the 5' position of the corresponding DR element and that this binding polarity of the DBDs is conferred upon the full-length receptors.

THE ADDITION OF A NUCLEAR MATRIX ASSOCIATED REGION PROMOTES THE BIOLOGICAL EFFECTS OF HPV11

Mo - 51

Christian Seelos and Christa Cerni

Institute of Tumorbiotherapy-Cancer Research, University of Vienna, Austria

Human papillomaviruses (HPV) are a group of small DNA viruses found associated with benign and malignant proliferative diseases originating from mucosal and keratinized epithelia. While much emphasis has been put in the last years on the molecular biological mechanisms by which high-risk HPVs (e.g. HPV type 18) contribute to cellular transformation, little attention has been paid to low risk types such as HPV 11 which only occasionally induce malignancies. We have previously shown that HPV11, despite contrasting reports from other laboratories, has a low but definitive immortalizing capacity *in-vitro* as shown by transfection of primary rat embryo cells (RECs). To define the mechanisms of this low immortalizing potential we are investigating the role of the upstream regulatory region (URR) of HPV11 in directing the expression of the viral early genes. Expression of transfected genes is not only determined by gene-intrinsic and/or cellular factors but varies with the chromosomal site of insertion as well. It has been shown, that cis-acting sequences that mediate the anchorage of DNA to the nuclear matrix (MAR) can significantly elevate the expression of reporter genes in stably transfected cells by conferring independence of chromosomal position. We show that, in contrast to wild-type HPV11, addition of a MAR 5' to the URR of HPV11 leads to a high number of immortalized cell-clones. Moreover, the sensitivity of the cell system to factors modulating the biological effects of HPV11 (e.g. cotransfection with c-fos) is markedly increased. Our construct might thus be used as a model to study the oncogenic potential of weak immortalizing or transforming genes.

SUPPRESSION OF TUMOR CELLS
PROLIFERATION BY SUBSTANCES DERIVED
FROM EPITHELIOCHEMIAL PLACENTA
TROPHOBlast

Mo - 52

R. Georgieva, D. Stefanov and G. Drjanovski,

Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Science, Sofia, Bulgaria

The effect of chromatographically isolated trophoblast substances (TS) from epitheliochemial placenta with Mr 60-70 kD on *in vitro* spontaneous proliferation of human erytroleukemic cells (K 562) as well as on development of transplantable Lewis lung cancer (LLT) in mice were studied. The 4×10^4 K 562 cells were cultured with 100 µg protein of TS in a final volume of 200 µl for 24 and 48 hrs. Thymidine (1 µ Ci) was added and DNA synthesis was recorded by RIA 6 hrs later. The 10^6 LLT cells in 100 µl hanks were injected into femoral muscle of C57BL/6 mice and TS at a doses of 100 µg protein was administered i.p. The diameter of the tumors was measured at 48 hrs intervals during 40 days.

The results showed that the TS inhibited strongly the proliferation of K 562 cells (91.1%) and there was no difference in 24 and 48 hrs cultures. TS suppressed significantly the development of LLT tumors ($P < 0.001$) and the effect was observed mainly when TS was injected before the tumor cells. A regression of the tumors was observed with time advancing.

Mo - 53 CARCINOGENS SPECIFICALLY DOWN-REGULATE PROTEIN KINASE C-ε IN C₃H/10T1/2 FIBROBLASTS

Z. Kiss and W. H. Anderson
The Hormel Institute, University of Minnesota,
Austin, Minnesota 55912 USA

The epsilon isoform of protein kinase C (PKC) was shown to be an oncogene when overexpressed in fibroblasts, suggesting that this enzyme may play a role in carcinogenesis. PKC-ε was also considered to be a regulator of phospholipase D (PLD). To verify these possibilities, we examined the effects of chemical carcinogens on the PKC and PLD systems in the C₃H10T1/2 mouse embryo fibroblast line, an established cellular model for the study of carcinogenesis. Treatment of fibroblasts with benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene (0.5 µg/ml of each) for 24 h resulted in nearly complete down-regulation of PKC-ε, while the other (α, δ, and ζ) PKC isoforms were unaffected. However, both carcinogens promoted phorbol ester-induced membrane translocation of cytosolic PKC-α and PKC-δ. Chronic (24 h) treatments with carcinogens specifically enhanced (1.5 to 2-fold) the hydrolysis of phosphatidylethanolamine but they failed to prevent phorbol ester activation of PLD. Similarly, up to 15-fold overexpression of PKC-ε in NIH 3T3 fibroblasts did not significantly modify the stimulatory effect of phorbol ester on PLD activity. The results suggest that in these fibroblasts, PKC-ε does not play a major role in either chemical carcinogenesis or in the regulation of PLD.

[Supported by the Hormel Foundation]

EGF RECEPTORS ON ROUS SARCOMA VIRUS-
Mo - 55 TRANSFORMED CELLS

V. Sovová, H. Fiedlerová and E. Šloncová

Institute of Molecular Genetics, Academy of Sci of the Czech Republic, Prague 6, Czech Republic

Epidermal growth factor (EGF) binding to hamster and mouse cells transformed by Rous sarcoma virus (RSV) was studied. It was found that EGF binding to tumor cells differed in mice and hamsters. While EGF binding was considerably reduced in mouse tumors, the binding activity of hamster cells did not change after RSV transformation.

ONCOGENES AND ANTIONCOGENES IN SARCOMAS OF CHILD AGE

Mo - 54 J. Mares^a, R. Kodet^b, V. Smelhaus^c, P. Goetz^a

^aInstitute of Biology and Medical Genetics, 2nd Medical Faculty, Charles University, Prague, Czech Republic, ^bInstitute of Pathological Anatomy, 2nd Medical Faculty, Charles University, Prague, Czech Republic, ^cClinic of Children's Oncology, 2nd Medical Faculty, Charles University, Prague, Czech Republic

Mutations or expressions of products of mutated oncogenes and antioncogenes have been studied in many adult malignant diseases, however a complex study of tumor gene mutations of pediatric solid tumors has not been done. In this preliminary study we present results concerning oncogene and tumor suppressor gene amplifications and deletions in children's sarcomas. The primary tumor tissues from 32 children's patients were investigated by a battery of 6 different probes (Rb, p53, v-ras, N-myc, v-erbA and v-erbB). We observed amplification of N-myc (18%), v-erbA (12%) and v-erbB (18%) and deletions of p53 (6%) and Rb (6%). The correlation of the results with cytogenetic and histological data and with clinical progression is discussed with regard to prognostic significance of analysis. The study contributes to the characterization of the malignant sarcoma and its progression in children's patients.

TRANSFORMATION OF MAMMALIAN CELLS BY

Mo - 56 MN ONCOGENE

S.Pastoreková, M.Vančíková, A.Gibadulina, K.Tarábková, D.Cmarko, Z.Závadová, O.Machon, J.Závada and J.Pastorek
Institute of Virology, Slovak Academy of Sciences Bratislava, Slovakia; ¹Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czechia

Several lines of evidence suggest that the protein MN, recently identified in HeLa cells is involved in oncogenesis. Expression of MN correlates both with density of HeLa cells and with tumorigenicity of HeLa x fibroblast hybrids. Moreover, MN protein is present in some human tumors, but not in corresponding normal tissues.

To address the key question, whether MN is an oncogene or only a tumor biomarker, we transfected the MN cDNA into mammalian cells and followed biological consequences of its expression. In NIH 3T3 cells, expression of MN resulted in changes that are indicative of malignant transformation. MN-transfected cells gained refractile spindle-shaped morphology with chaotic growth on top of one another. Consistently with the transformed phenotype, the cells expressing MN showed increased saturation density and reduced growth factor requirements. Finally, they displayed anchorage-independent growth in soft agar, which is known to correlate well with the tumorigenicity.

In view of these data we propose that the MN protein is the product of a new oncogene. At present, MN expression in other cells and study of its tumorigenic potential *in vivo* are underway.

MN - A NOVEL TYPE OF ONCOPROTEIN

Mo-57

J. Pastorek, R. Kejtmann¹, S. Pastoreková, I. Callebaut, J.P. Morin², R. Opavský, V. Zelník, A. Burny³ and J. Závada⁴

Institute of Virology, Slovak Academy of Sciences Bratislava, Slovakia; ² Faculty of Agronomy, Gembloux, Belgium; ³ Institute of Molecular Genetics, Prague, Czechia; Universities of Pierre and Marie Curie and Denis Diderot, Paris, France

We have cloned and sequenced full-length cDNA coding for a novel oncoprotein MN that is expressed in several human tumors and has a transformation potential *in vitro*. According to Southern blot analysis, the MN gene is conserved in a single copy in DNA of vertebrates. Northern blotting confirmed that the expression of MN is density-dependent and tumorigenicity-related. Amino acid sequence of MN deduced from the cDNA contains consensus sequences consistent with the findings that MN is glycosylated and localized both in plasma membrane and nucleus. Hydrophobic cluster analysis revealed strong homology between the central region of MN and carbonic anhydrase, with the conserved zinc-binding site and enzyme's active center. The N-terminal part of MN shares some similarity with helix-loop-helix proteins. The region between these two domains is rich in imperfect repeats of Ser, Pro, Gly and acidic residues, resembling thus an activation region of transcription factors. Taken together, the features of MN suggest that it represents a potentially novel type of oncoprotein.

REGULATION OF MN EXPRESSION

Mo-58

R. Opavský, S. Pastoreková, M. Vančíká, S. Kaluz, J. Lieskovská, P. Kabát, A. Čadulinová², A. Mills¹, E.J. Stanbridge², Z. Závadová², J. Závada² and J. Pastor¹

Institute of Virology, Slovak Academy of Sciences Bratislava, Slovakia; ² University of California Irvine, USA; Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czechia

MN is a novel human protein that appears participate in oncogenesis. In HeLa cells, the expression of MN is positively regulated by cell density. Its level is also increased by persistent infection with LCMV. In hybrid cells between HeLa and normal fibroblasts, MN expression correlates with tumorigenicity. The fact that MN is not present in nontumorigenic hybrid /NT/, but is expressed in tumorigenic segregant lacking chromosome 11, indicates that MN is negatively regulated by a putative suppressor in chromosome 11.

To study MN regulation, MN genomic clone was isolated from human fetal brain library. Sequence analysis revealed that MN promoter is GC-rich and contains one putative TATA-box 578bp upstream the transcription start. In addition, it possesses several consensus sequences for binding sites of regulatory elements: tandem of p53 sites, AP-1/2, SP-1, SDRE sites etc. At present, the promoter clone is subjected to further analyses.

When the promoter region was linked to MN cDNA and transfected into NT hybrids, expression of MN protein was detectable immediately after selection. However, then it gradually ceased, indicating thus an action of a feedback loop.

TRANSFORMATION BY RETROVIRAL ONCOGENES
INTERFERES WITH MUSCLE CELL DIFFERENTIATION
Mo-59 BY MULTIPLE MECHANISMS

Simona Russo, Milena Grossi & Franco Tardini

Dip. Biologia Cellulare e dello Sviluppo, Università di Roma "La Sapienza", Rome, Italy.

Skeletal myogenic cells *in vitro* represent an attractive system to study cell differentiation and the relationship between transformation and terminal differentiation. Transformation of avian myogenic cells can be achieved with a single oncogene and the establishment of the transformed state prevents to a large extent terminal differentiation. Following the identification of the factors involved in muscle determination and differentiation, the understanding of the mechanism by which oncogenes can interfere with muscle differentiation has considerably progressed.

We have infected primary quail myoblasts with retroviruses carrying oncogenes belonging to all functional groups. The same overall phenotype, transformation with block of differentiation, is elicited by oncoproteins endowed with widely different biochemical properties, and residing in different compartments of the cell. Several oncogenes suppress to variable extent the accumulation of transcripts for the myogenic regulatory factors MyoD and Myogenin.

We have also studied the forced expression of exogenous myogenic regulatory factors in primary quail myoblasts transformed by the ras oncogene. The results will be shown and discussed at the poster session.

Supported by Fondazione Cenci-Bolognetti, AIRC (Progetto Oncosoppressori) and CNR-PF-ACRO.

MYC ONCOGENE AND MYOGENESIS:
ANALYSIS OF TRANSFORMATION AND
BLOCK OF DIFFERENTIATION

S.A. La Rocca, D.H. Crouch*, D.A.F. Gillespie* and F. Tardini

Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Roma "La Sapienza", Via degli Aprili 1, 00185 Rome, Italy; *Beatson Institute for Cancer Research, Garscube Estate, G61 1BD, Glasgow, Scotland.

Myogenesis represents a particularly attractive system for studying the genetic basis of differentiation and the relationship between proliferation and terminal differentiation. We have analysed the interference of a variety of oncogenes with the differentiation program of avian primary myoblasts. *In vitro* transformation of primary myoblasts by the oncogenes *ras*, *src*, *sea*, *mil*, *myc*, *fps*, *jun* and *fos* is accompanied by inhibition of terminal differentiation.

The discovery of the muscle-specific regulatory factors (MRF), known as the MyoD family, has facilitated the understanding of the mechanism by which oncogenes can interfere with muscle differentiation. The analysed oncogenes interfere with the expression of the MRFs, they inhibit one or more members of the MyoD family. Among all the others we have particularly studied the *myc* oncogene. While in C2C12 myogenic cell line the constitutive expression of viral or cellular *myc* blocks the fusion but not the terminal differentiation, in *myc*-transformed primary avian myoblasts not only the terminal differentiation is blocked but is also inhibited the expression of the MRFs. We show in our studies that the *myc*-induced block of myogenic differentiation in primary avian myoblasts is associated with the absence of muscle specific gene products, such as myosin, and reduced expression of the positive myogenic regulatory factors of the MyoD family. In further studies mutant alleles in different Myc domains are used to clarify the mechanism through which the oncogene inhibits myogenic differentiation. Particularly Myc mutants in the Leucine Zipper domain (LZ), which impair or abolish Max (Myc associated protein) dependent transcriptional activation, are tested for their ability to transform and inhibit the terminal differentiation.

Supported by Fondazione Cenci-Bolognetti, CRC, AIRC (Progetto Oncosoppressori) and CNR-PF ACRO.

Mo - 61 ALTERATIONS IN PROTEIN SYNTHESIS
INDUCED BY MICROFILAMENT

DEPOLYMERIZATION IN 3T3 CELLS

Oleg Denisenko, Tatyana Sklyarova, Vadim Sharov,
Vladimir Kostyukovskiy*, & Victor Prisyazhnov†

Institute of Protein Research, Russian Academy of Sciences, * Institute of Biochemistry and Physiology of Microorganisms, †Branch of the Institute of Bioorganic Chemistry, 142292 Pouschino, Moscow Region, Russia;

C2 toxin was purified to homogeneity from culture medium of *Clostridium botulinum* type C. The toxin preparation specifically ADP-ribosylated actin *in vitro*. ADPR-actin changed its pI to a more acidic on 2D-gels, this phenomena was used to monitor the extent of actin modification *in vivo*. The toxin was used to depolymerize actin filaments in 3T3 cells. After 4 hours of treatment with C2 toxin the cells were rounded off, and detached from the substrate. This was accompanied by a complete disappearance of stress fibers in the cells. The effect of actin filaments depolymerization on protein synthesis was studied. The rate of total protein synthesis decreased two times in the toxin treated cells. It was correlated with the two-fold reduction of the polyribosomes quantity and a concomitant increase of the monoribosomes. The rates of specific proteins synthesis were compared using 2D electrophoresis of pulse-labelled proteins. Cytoskeletal actin synthesis was shown to be significantly reduced in response to C2-toxin treatment. Changes were also observed in the synthesis of some other cellular proteins. Total RNA preparations from control and toxin-treated cells were translated *in vitro*, the translational products were analysed by 2D electrophoresis. These results show that the synthesis of some proteins was changed in response to microfilaments depolymerization due to variations in the mRNAs quantities, but synthesis of others was altered predominantly at the translational level.

Mo - 63 THE BASIC CELLULAR MECHANISMS
INVOLVED IN LIVER FIBROSIS

Ch. M. Lapierre and B.V. Nusgens

Laboratory of Experimental Dermatology, CHU,
Sart Tilman par Liège 1, 4000 Liège, Belgium

Several forms of liver diseases evolve towards a fibrotic reaction characterized by accumulation of extracellular proteins, disruption of the organization of the tissue and further impairment of its function. Injury of liver cells by toxic compounds or viruses induces the release of cytokines responsible for the changing properties of endothelial cells, migration of blood borne cells and differentiation of resident cells in activated fibroblasts and myofibroblasts. The phenotypic properties of such cells can be investigated *in vitro*. Myofibroblasts retract a collagen gel more efficiently than fibroblasts and produce less collagenase. Investigation of the mechanisms operating in the control of cell phenotype *in vitro* suggests that the matrix integrin mediated signals are more efficient than those depending on cytokines. Intracellular signalling begins to be known and involves the cytoplasmic parts of both α and β sub-units of integrins, protein tyrosine kinases, phospholipases, metabolites of phospholipids, Ca^{++} , oncogenes and cytoskeletal proteins. Such investigations could lead to discovery of specific therapies for various types of fibrosis and support already used treatments as, for example, interferons and colchicine.

Mo - 62 FACTORS INFLUENCING THE CYTOSKELETAL FEATURES OF FIBROBLASTS.

A. DESMOULIERE ^{a,b}, and G. GABBIANI ^a

^a University of Geneva, Department of Pathology,
CMU, 1, rue Michel-Servet, 1211 Geneva 4,
Switzerland; ^b CNRS - URA 1459, Institut Pasteur de Lyon,
avenue Tony Garnier, 69365 Lyon cedex 7, France

Granulation tissue fibroblasts (myofibroblasts) develop several ultrastructural and biochemical features of smooth muscle (SM) cells, including the presence of microfilament bundles and the expression of α -SM actin, the actin isoform typical of contractile vascular SM cells. During wound healing, α -SM actin expressing myofibroblasts disappear when the wound is fully epithelialized. In contrast, the expression of α -SM actin is a more permanent feature of myofibroblasts present in fibrocontractive diseases. The mechanisms leading to the development of myofibroblastic features remain to be explored. In vivo and *in vitro* investigations have shown that γ -interferon develops an antifibrotic activity by decreasing α -SM actin expression whereas heparin increases the proportion of α -SM actin positive cells. The subcutaneous administration of transforming growth factor- β 1 (TGF β 1) results in the formation of a granulation tissue in which α -SM actin expressing myofibroblasts are particularly abundant. Furthermore, TGF β 1 induces α -SM actin protein and mRNA expression in growing and quiescent cultured fibroblasts. These results suggest that TGF β 1 plays an important role in myofibroblast differentiation by regulating the expression of α -SM actin in these cells. Further studies on the expression of cytoskeleton markers in fibroblastic cells could be useful for the understanding of the mechanisms of development and regression of pathological processes, such as wound healing and fibrosis.

Mo - 64 ROLE OF IL-1 β AND TNF α CYTOKINES IN PUROMYCIN AMINONUCLEOSIDE NEPHROSIS

A. Martín, Molina A., Bricio T. and
Mampaso F.

Department of Pathology, Hosp. Ramon y Cajal,
Madrid, Spain.

The administration of a single dose (10 mg/100gr b.w., i.p.) of aminonucleoside of puromycin (PAN) to rats produces elevated proteinuria and changes in the ultrastructure and function of glomerular cells. Both IL-1 β and TNF α concentrations from PAN-treated rat cultured glomeruli supernatants, showed a significant increase ($P<0.01$) during the first two weeks of disease (IL-1 β : 10500 c.p.m., control: 1300 c.p.m.; TNF α : 50-70 pg/ml, control: 20-28 pg/ml). *In vivo* treatment of PAN-induced nephrotic rats with anti-TNF α (100.000 I.U., n=5), and anti-IL-1 β (100 ug, n=5) as well as with both antibodies simultaneously (n=5) provoked a significant reduction in the levels of proteinuria in all treated rats, being this reduction higher when both cytokines were used together.

PROTEINURIA (mg/24h)	Control	PAN	α -IL-1 β	α -TNF α	α -IL-1 β +TNF α
day 7	5±0.5	25±8.4	95±5.1	127±8	68±4.3
day 14	4±2.6	173±52	58±4.4	127±50	36±21
day 21	3±1.4	14±9	20±9	19±8	26±12

Our results demonstrated that both IL-1 β and TNF α cytokines are important mediators in the glomerular filtration barrier dysfunction, thus favoring the development of proteinuria in this model of experimental nephrosis.

DAMAGED HEPATOCYTES PROMOTE MITOGENIC ACTIVATION OF CULTURED RAT LIVER FAT STORING CELLS

MO - 65 A.M. Gressner and Ch. Hoffmann, Dept. of Clinical Chemistry and Central Laboratory, Philipps University, 35033 Marburg

Introduction: Mitogenic stimulation of fat storing cells (FSC), the principal matrix producing cell type in liver, is recognized as the key process in hepatic fibrogenesis. We put forward the hypothesis that besides established paracrine mechanisms due to inflammatory cells, hepatocytes (PC) might also be involved in this process since (i) PC are intimately connected with FSC in situ, (ii) PC injury frequently precedes and accompanies FSC activation in the tissue.

Methods: PC were isolated by the collagenase method. Controlled cell damage was induced by addition of carbonyl-cyanide m-chlorophenyl-hydrazone (CCCP, 2 µmol/l), t-butylhydroperoxide (TBHP, 0.2 mmol/l), and Ca-ionophore A23187 (20 µmol/l), respectively, during collection of PC-conditioned media (PCcM) for 24 h. LDH, AST, and total protein were determined in PCcM before dialysis against DMEM. Various dilutions of PCcM were added to nonconfluent FSC monolayers in 0.5% FCS to measure [³H] thymidine incorporation into DNA.

Results: In parallel with the decrease of PC viability, mitogenic activity of PCcM increased significantly up to 3 fold of control. TBHP and A23187 (but not CCCP) greatly damaged PC monolayers, but increased simultaneously the mitogenic potency of the respective PCcM for FSC significantly. Mitogenic activity was positively and significantly ($p < 0.05$) correlated with LDH ($r = 0.88$), AST ($r = 0.94$), and total protein ($r = 0.82$) in PCcM.

Discussion: These results indicate that PC damage significantly stimulates FSC proliferation in vitro. The effect might be mediated either by release of a mitogen from a cytosolic storage site ("wound hormone") or by decrease of an inhibitor normally secreted by PC. This mechanism might be important for the preinflammatory activation of FSC in damaged liver.

MO - 67 MOLECULAR ASPECTS OF THE CROSS-TALK BETWEEN PERISINUSOIDAL LIPOCYTES, PARENCHYMAL AND NON-PARENCHYMAL CELLS IN LIVER FIBROGENESIS

A.M. Gressner

Department of Clinical Chemistry and Central Laboratory, University Hospital, 35043 Marburg, Germany

Activation of perisinusoidal lipocytes (PL), i.e. mitogenic stimulation, transformation, and matrix gene expression is recognized as the critical event in initiation of liver fibrogenesis. Recent studies of our and other laboratories have established main cell and molecular biological reactions contributing to a cascade mechanism of PL activation. Parenchymal cell (PC) damage is likely to be the first, *preinflammatory step* of PL activation since mitogenic factor(s) are released ("wound hormone") of which the molecular identification is currently in progress. The stimulator acts synergistically with proinflammatory cytokines released by activated Kupffer cells (see below). In addition, the disappearance of constitutively expressed growth inhibitors in damaged PC contributes to PL deactivation (= stimulation). In a second, *inflammatory phase* influx of MΦ, activation of Kupffer cells (KC), and disintegration of platelets release profibrogenic cytokines including TGF β 1, TGF α , TNF α , IL-1 and others, which upregulate activation of PL. In the third, *postinflammatory step* autocrine growth stimulation of transformed PL involving TGF β , FGF and others might contribute to self-perpetuation of fibrogenesis even after cessation of the primary events. The interplay between cytokines and cells is critically influenced by the changing extracellular matrix (ECM) having multiple binding sites for growth factors. In addition, modulators of cytokine activity like α_2 -macroglobulin produced by PC and PL, respectively, greatly affect extracellular availability of growth signals. Liver fibrogenesis turns out to be a highly interesting paradigm of the pathogenetic significance of cellular cross-talk in disease processes.

MYOFIBROBLAST GENERATION FROM HUMAN BREAST FIBROBLASTS

MO - 66 Lone Rønnow-Jessen and Ole W. Petersen

Laboratory of Tumor Endocrinology, the Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen Ø; and Structural Cell Biology Unit, Institute of Medical Anatomy, the Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

Alpha-smooth muscle actin is expressed in peritumoral stromal cells (myofibroblasts) of human breast carcinomas. We have sought the cellular source of these cells with a view to identifying the cytokine(s) responsible for induction of alpha-smooth muscle actin. We set out to describe the stromal cells of the normal breast *in situ* and in culture, and a method for the purification of fibroblasts from normal breast specimens was developed. However, we soon learned that in the presence of serum, alpha-smooth muscle actin was induced in more than 80% of the isolated fibroblasts within 3-5 days of cultivation. Therefore, an experimental system maintaining the normal *in situ* fibroblast phenotype seemed warranted.

A serum-free culture model allowing fibroblasts to stay quiescent but at the same time sensitive to environmental cues was developed. A panel of cytokines was tested for their ability to induce alpha-smooth muscle actin in fibroblasts under these conditions. Only TGF-beta1 was found to have this capacity. The effect of TGF-beta1 was dose-dependent with a maximal response above 80 pg/ml and a course of 6 days. Selection of alpha-smooth muscle actin-positive cells could be excluded since no proliferation was observed within the experimental period. Most importantly, a similar response was obtained upon stimulation with serum-free conditioned medium from breast carcinoma cells. Preincubation with anti-TGF-beta antibodies neutralized this effect. In conclusion, our data suggest that breast tumor cells generate myofibroblasts from normal breast fibroblasts by paracrine stimulation.

References:
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Rønnow-Jessen, L. & Petersen, O. W. Lab. Invest. 68:696-707, 1993

THE *IN VITRO* EFFECTS OF EGF C-LOOP (CVIGYSGDRC-NH₂) AND THE LAMININ B1 (CDPGYIIGSR-NH₂) PEPTIDES ON THE ATTACHMENT AND MIGRATION OF ENDOTHELIAL CELLS

MO - 68 W.E. ALLEN, W.N. SCOTT*, J.R. BAILIE¹, D.J. WILSON, B. WALKER* and J. NELSON*. Schools of Biomedical Science, Biology and Biochemistry* & Clinical Medicine¹. The Queen's University of Belfast, N. Ireland.

Angiogenesis has attracted much interest because of its role in many pathological conditions, particularly solid tumour growth. Consequently research has focused on factors which regulate angiogenesis, particularly the role of growth factors (e.g. EGF and TGF- α) and basement membrane proteins such as laminin. We have investigated the angiogenic properties of two highly homologous synthetic peptides: a linear decapeptide from the C-loop of EGF and a linear nonapeptide from the B1 chain of laminin using two *in vitro* assays, a phagokinetic track migration assay and an attachment assay using a laminin substratum. The migration assays were carried out using embryonic chick endothelial cells, bovine retinal capillary endothelial cells and a transformed human line of endothelial origin (SK-HEP-1, obtained from the ECACC, Porton Down, Salisbury, UK). The chick cells and T-47D human breast cancer cells (used because of the role of laminin in tumour cell attachment and during metastatic spread) were used for the attachment assay. Both the cells used produced similar results in this assay, with 50% inhibition of attachment occurring with 250 nM concentrations for native laminin, the B1 peptide and the C-loop. The migration assay results showed that with the B1 peptide track area doubled over control at a dose of 50 nM, while with the C-loop it was halved in comparison with the control at 50 nM. Competition results show that EGF C-loop antagonises the action of laminin and EGF in the migration assay, and that the effects of the laminin B1 peptide enhance those of EGF and laminin.

THE EXPRESSION OF THE CD4-LIKE
MOLECULES ON HUMAN SPERMATOZOA

Mo - 69

D.K.Dimitrova-Dikanarova, Ts.Ts.Marinova, R. Fichorova

Laboratory for Reproductive Immunology, Department of Biology,
Medical Faculty, Sofia, Bulgaria

Human spermatozoa express a large number of membrane antigens involved in male subfertility, some of them expressed on other cells, including T-Lymphocytes.

In our experiments the expression of molecules, recognized by CD4 monoclonal antibodies, was investigated on human spermatozoa (normal and round-headed) and thymocytes using ELISA, indirect immunofluorescence, and immunogold staining methods.

The results demonstrated the presence of CD4 immunopositive spermatozoa and thymocytes. CD4-like antigens were surface localized in thymocytes and sperm, mostly on the equatorial segment and the postacrosome. Immunofluorescence was detected on round-headed sperm.

These findings suggest that the expression of CD4-like molecules on spermatozoa might be associated with male subfertility.

THE MOLECULAR BASIS OF THE SPERM
INFERTILITY

Mo - 70

H. M. Gulaya and V. M. Marvitich

Department of biochemistry of lipids,
Institute of Biochemistry. Leontovich str. 9,
252030. Kiev, Ukraine.

Altered lipid metabolism is often associated with defects of many membrane bound function. It is well known that the fertility depend on the adequate gamet membrane function.

The Phospholipid composition of spermatozoa from healthy and infertile men was studied by two dimensional micro thin layer chromatography.

It was found that spermatozoa from infertile men showed the drastique loss of Phosphatidyl ethanolamine. Its amount decreased from the 25% by the 5% of total Phospholipid phosphorus. On the other hand, the significant increase (from 7% by 21%) of Phosphatidyl serine was found. It is interesting to note that both phospholipids are localized mainly in the inner side of the plasma membrane. Simultaneously, the amount of sphingomyelin was found to be increased. It was also shown that very high amount (above 5% of total phospholipids) of lyso-phosphatidylserine is present in the spermatozoa of the most part of infertile men. In normal spermatozoa lyso-phosphatidylserine generally was not detectable.

These data suggested that one of the reasons of infertilility can be a spermatozoa disease caused by the alteration of its phospholipid composition.

EFFECT OF CHRONIC HYPOXIA ON FATTY
ACID COMPOSITION IN MYOCARDIAL
PHOSPHOLIPIDS DURING ONTOGENESIS

Mo - 71

O. Nováková^a, V. Pelouch^b,
E. Tvrzická^c, D. Smík^a and F. Novák^d

^aDepartment of Animal Physiology,
^dDepartment of Biochemistry, Faculty of Natural
Sciences ^cAngiol. Laboratory of First Medical
Faculty, Charles University, ^bInstitute of Phys-
iology, Czech Academy of Science, Prague,
Czech Rep.

The male Wistar rats four-days old were exposed to intermitent high altitude (IHA) hypoxia in barochamber (7000m, 8 hrs a day, 5 days a week, 24 expositions). IHA induced hypertrophy of right ventricle (RV) by 32% and left ventricle (LV) by 20%. Except of diphosphatidyl-glycerol (DPG) decrease in LV, no change in concentration of any phospholipid species was found after IHA treatment. IHA induced alteration in fatty acyl (FA) composition of phospholipids in both ventricles: the content of 18:0, 20:4 (n-6), 22:5 (n-3), 22:6 (n-3) increased whereas amount of 18:1 (n-9), 18:2 (n-6) decreased in phosphatidylcholine. In phosphatidyl ethanolamine the content of 18:0, 22:5 (n-3), 22:6 (n-3) increased and 18:6 (n-6) decreased. The fraction of DPG retained its high amount of 18:2 (n-6) and the content of monounsaturated FA increased. 30 days after returning into normoxic conditions hypertrophy still lasted whereas FA composition reverted to control values. After 100 days in normoxia the complete regression of hypertrophy to control level was observed.

DNA LABELLING INDEX VALUES FOR COLON IN
PATIENTS AT HIGH RISK OF COLORECTAL
CARCINOMA

M.Kment^a, F.Kocourek^b, V.Mares^{b,c}, R.Pytlik^a

^a2nd Department of Medicine, 3rd Medical Faculty, Charles
University, Prague, Czech Republic; ^bInstitute of
Physiology, Academy of Sciences of the Czech Republic, Prague; ^cDept.of
Zoology, Academy of Sciences of the Czech Republic, Prague, Kuwait

The causes of increase in the incidence of colorectal carcinoma are not clear. Current studies investigate possible protective influence of different drugs e.g. calcium. The proliferation of colon cells is most frequently monitored by DNA synthesis revealed by incorporation of BrdU or ³H-thymidine in cells in the S phase of the cell cycle i.e. the Labelling Index values (L.I.) measured in *in situ* or *in vitro* labelled tissue. According to previous data, colon L.I. values greatly vary. In mice and rats, the values range from 6.1% to 34.7%. The variations may depend on the choice of proliferation marker (BrdU, ³HTdR, PCNA), the way of administration of the DNA precursor and the part of the colon actually studied.

The aim of our study was to assess colon L.I. in the group of patients with high risk for colorectal carcinoma. We used the method of ³HTdR labelling of biopsy samples obtained from the colon of 16 patients who were then monitored for a period of 6 month. To examine an impact of penetration of ³HTdR into the biopsy we either cut the samples into very small fragments or stretched the mucosa on a filter paper support. The samples were incubated in MEM with 10% Bovine Serum and ³H-thymidine (2μCi/ml media) at 37°C, 5% CO₂ for 120 min. The average value of LI found in our material was 11.03% and SD=1.3%. The values obtained from the sliced and stretched incubated samples gave comparable values of L.I. 10.84% ± 1.08% and 9.55% ± 0.47%, respectively. In 6 patients, similar L.I. values were found in the material sampled repeatedly over the 6 month period while in 2 patients the values were more variable (6.12% to 20.85%). However, the rate of proliferation in the human colon in the *in vitro* conditions was found similar to that reported earlier and it appeared closer to the values obtained under *in situ* conditions reported e.g. for rat.

Mo-73 LIPID PEROXIDATION IN ISOLATED MEMBRANES AND ITS EFFECT ON THE ACTIVITY OF VARIOUS ATPASES

H. Rauchová^a, M. Kalous^a, Z. Drahota^a, J. Koudelová^b and J. Mourek^b

^aInstitute of Physiology, Academy of Sciences of the Czech Republic; ^bInstitute of Physiology, First Faculty of Medicine, Charles University, Prague, Czech Republic

The extent of enzymatic and non-enzymatic induced peroxidation measured as production of thiobarbituric acid-reactive substances (TBARS) was determined in isolated membranes from cerebral cortex, heart and kidney of 21-day-old Wistar rats. Our aim was to evaluate the extent of lipid peroxidation in the absence of endogenous protecting systems present in the cytosol. The time course of induced peroxidation showed higher production of TBARS in cerebral cortex than in heart or kidney.

The activity of various ATPases localized on renal membranes was measured after 10 min incubation with lipid peroxidation inductors (ADP.Fe/NADPH). We found that Mg²⁺-dependent ATPase activities (ouabain-sensitive plasmatic Na⁺,K⁺-ATPase and other Mg²⁺-dependent ATPases, e.g. mitochondrial H⁺-ATPase) were decreased whereas Mg²⁺-independent ATPase activity was increased. The similar results were also obtained for brain membrane-bound ATPases. This indicates different mechanisms through which lipoperoxides affect the function of membrane-bound enzymes.

Mo-74 PROTECTIVE EFFECT OF CARNITINE ON HYPOXIA-INDUCED LIPID PEROXIDATION IN THE RAT BRAIN CELLS

J. Koudelová^a, J. Mourek^a, Z. Drahota^b and H. Rauchová^b

^aInstitute of Physiology, First Faculty of Medicine, Charles University; ^bInstitute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Our previous experiments revealed high lipoperoxide formation induced by hypobaric hypoxia in brain cells of 21-day-old Wistar rats (Koudelová and Mourek: *Physiol. Res.* 41, 207-212, 1992).

Our aim was to evaluate the effect of carnitine treatment on the extent of lipid peroxidation induced by 30 min hypobaric hypoxia (corresponding to 9000 m). The content of thiobarbituric reactive substances (TBARS) was determined in cerebral cortex, subcortical structures, medulla oblongata and cerebellum. Control and carnitine treated animals were compared. The carnitine administration (80 mg/kg, intraperitoneally, 30 min before hypoxia) significantly lowered TBARS production in brain cells of all above mentioned areas. The enhanced TBARS production by hypobaric hypoxia was thus completely abolished.

Mo-75 COENZYME Q (CoQ) PROTECTS HEPATOCYTE MITOCHONDRIA FROM ADRIAMYCIN-INDUCED OXIDATIVE STRESS

M. Cavazzoni^a, J.E. O'Connor^b, T. Ruffolo^a, V. Valls^b, G.T. Saez^b and G. Lenaz^a

^aDept. Biochemistry, University of Bologna, Italy; ^bDept. Biochemistry and Molecular Biology, University of Valencia, Spain

Adriamycin is a potent drug in cancer therapy, but its clinical use is limited by its severe toxicity, whose mechanism involves the reduction of adriamycin to the semiquinone, followed by redox cycling in the presence of molecular oxygen and release of superoxide and H₂O. We have used adriamycin to induce an oxidative stress in isolated rat hepatocytes, and we have studied the effects on the mitochondrial function "in situ".

The incubation with 25 μM adriamycin increases the amount of hydrogen peroxide as determined by 2',7'dihydro-dichlorofluorescein-diacetate: the flow-cytometric analysis shows two subpopulations of rat hepatocytes that have a different ability to counteract the drug.

In the presence of adriamycin we have observed a decrease of the respiratory activity and the total amount of endogenous CoQ₁₀ in isolated rat hepatocytes. The mitochondrial membrane potential is a key parameter to investigate the metabolic and energetic state of the cell; we have evaluated it using the fluorescent probe Rhodamine-123 (Rh-123) and flow-cytometry. The adriamycin treatment was also found to strongly affect the mitochondrial membrane potential. Incorporation of CoQ₁₀ in isolated hepatocytes via liposomes preserves the CoQ₁₀ levels and protects the mitochondrial function, as tested by oxygen consumption and Rh-123 uptake, against the free radical attack induced by the drug. We conclude that CoQ preserves and potentiates the normal cellular defenses most probably through its antioxidant action.

Mo-76 CHANGES IN NEUTRAL AMINO ACID INFUX DURING THE REGULATORY VOLUME INCREASE OF HUMAN FIBROBLASTS

V. Dall'Asta, P.A. Rossi, O. Bussolati and G.C. Gazzola
Istituto di Patologia Generale, Università degli Studi di Parma, Via Gramsci 14, I-43100 Parma, Italy

Cultured human fibroblasts (CHF) counteract effectively cell shrinkage caused by the incubation in hypertonic medium (Dall'Asta et al., *J.Biol.Chem.*, *in press* (1994)). This regulatory volume increase (RVI) results from the enhanced accumulation of neutral amino acids, associated with a rapidly ensuing, long lasting cell hyperpolarization and a slower, protein-synthesis dependent increase in the capacity of transport system A. The influx of MeAIB, a site-A specific substrate, has been studied in CHF undergoing RVI. In comparison with control cells, maintained in isotonic conditions, CHF incubated for 30 min in hypertonic conditions exhibited a 15% higher MeAIB influx. The change was abolished if the hyperpolarization associated to the hypertonic treatment was suppressed by an increase in [K⁺]_{out}. On the contrary, neither cycloheximide nor actinomycin D affected the increase in MeAIB influx detected after 30 min of exposure to hypertonic medium. After 4 h of hypertonic treatment MeAIB influx was doubled; in this case, however, the increase was suppressed by cycloheximide and actinomycin. It is concluded that RVI of CHF results from the increase of neutral amino acid uptake through system A. Two distinct phases of the recovery process can be envisaged; at first, the increased accumulation of amino acids is accounted for by hyperpolarization alone; subsequently, both hyperpolarization and the protein synthesis dependent enhancement of system A capacity are responsible for the late increase of neutral amino acid influx through system A.

(Aided by CNR, Target Project ACRO, Rome, Italy)

Mo-77 THE MEMBRANE CONDUCTIVITY FLUCTUATIONS AS A MANIFESTATION OF THE EARLY STAGE OF ENVELOPED VIRUS ENTRY INTO THE CELL

A.E. Grinfeldt

Institute of Cytology of the Russian Academy of Sciences, 4 Tichoretsky avenue 194064 Saint-Petersburg, Russia

Initial events of penetration of the enveloped viruses into the cell cytoplasm include the virus attachment to the cell surface and fusion of the virus envelope with the cell membranes. These processes are triggered by virus envelope glycoprotein, and in particular by its N-terminal part named the fusion peptide. We revealed that the synthetic virus fusion peptides initiated the discrete fluctuations of conductivity of bilayer lipid membranes in many characteristics similar to those for ionic channels of cell membranes and for channels induced in lipid bilayers by incorporation of specific channel-forming substances. The induction of conductance fluctuations considered as a general characteristic feature of microlesion of the membrane. Then the fluctuations are available for indication of the crucially important stage of virus infection - membrane fusion preceding the entry of virus into the host cell and may be used for study of conditions preventing the fusion process.

Mo-79 MULTIDRUG RESISTANCE AS A CONSEQUENCE OF P-GLYCOPROTEIN OVEREXPRESSION IN L1210 MOUSE LEUKEMIA CELL LINE

Z. Štefanková^a, M. Barančík^a and A. Breier^b

^a Institute for Heart Research and ^b Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Dúbravská cesta 9, 842 33 Bratislava; Slovak Republic

A vincristine resistant cell line L1210/VCR was obtained from mouse leukemia cells L1210 by long-term adaptation in a medium with stepwise increasing concentrations of vincristine. Resistance of L1210/VCR cell line was found to be connected with the overexpression of drug transporting P-GP and cross resistance towards vinblastine, adriamycin and actinomycin D. Significant lack of capability to accumulate [³H]-vincristine was observed for L1210/VCR cells in comparison with non resistant L1210 cell line. This result agree with assumed ATP-dependent drug efflux activity of P-GP that is believed to be responsible for MDR. Multidrug resistance may be depressed by application of chemosensitizers, such as (1) calcium entry blockers (verapamil and nifedipine), (2) neuroleptics (trifluoperazine) and (3) local anaesthetics (lidocaine) directly to the grow medium. No reasonable correlation between the ability of calcium entry blockers to reduce the viability of L1210/VCR cells growing in the medium supplemented with vincristine and their reported affinity to the L-type of calcium channel was observed. On the other hand, significant positive correlations were observed between both: (i) the inhibitory action of local anaesthetics on propagation of action potential in rat sciatic nerve as well as (ii) the ability of drugs to interact with calmodulin with the ability of respective drugs to reverse the resistance of L1210 cells towards vincristine.

Mo-78 SACCHAROMYCES CEREVIAE MUTANTS WITH ENHANCED EXPORT OF PROTEINS

P. Venkov^a, K. Alexieva-Botcheva^a, M. Koprinarova^a, P. Segundo^b, F. del Rey^b and F. Klis^c

^a Institute of Molecular Biology, 1113 Sofia, Bulgaria

^b Instituto Microbiología Bioquímica, 37007 Salamanca, Spain, Dept Mol Cell Biology, University Amsterdam, 1098 SM Amsterdam, The Netherlands

Mutants temperature sensitive for enhanced protein export were selected among mutants giving positive staining for alkaline phosphatase at 37°C. Evidence were obtained to exclude cell lysis as a reason for the protein export. Genetic studies indicate that export mutants fall in two complementation groups and that one recessive nuclear mutation is responsible for the export of proteins in each mutant. Cultivated at 37°C the mutants export 25-30% of cellular proteins (versus 1% in parental strain) by an active process dependent on ATP and protein synthesis. The process takes place in rich medium only and is completely inhibited by cultivation the mutants in minimal medium or oxidative phosphorylation is impaired by rising rho derivatives from the export mutants. Exported are cell surface proteins and some of the proteins normally sorted to the vacuole or plasma membrane. Protein export in mutant cells starts after two cell divisions at 37°C which suggests dilution of a stable wild type product as possible reason for the enhanced export. Experiments are in progress to clarify if protein export in the mutants uses the secretory pathway or alternative routes.

Mo-80 *Candida albicans* TRANSPORT SYSTEM FOR BASIC AMINO ACIDS EXPRESSED IN *Saccharomyces cerevisiae*

H. Sychrová, A. Matějčková and A. Kotyk

Department of Membrane Transport, Inst. Physiology, Academy of Sciences of the Czech Republic, Prague 4, Czech Republic

Using a yeast/E. coli shuttle vector YEP352, the gene CAN1 coding in *C. albicans* for a permease specific for basic amino acids was expressed in *Saccharomyces cerevisiae*. Its expression showed that the permease actively transports across the plasma membrane arginine, lysine, histidine, as well as their toxic analogues canavanine and thialysine with high affinity. The intracellular concentration of basic amino acids transported into *S. cerevisiae* by the heterologous permease reaches more than a thousand-times-higher value compared to the external concentration in the medium. Accumulated amino acids do not leave the cells. The uptake is strongly reduced by the protonophores and inhibitors of plasma membrane proton ATPase.

Transport activity of the *C. albicans* permease CAN1 is in *S. cerevisiae* reduced by the presence of ammonium ions, whilst the *S. cerevisiae* own systems transporting basic amino acids (CAN1 and LYP1) are not ammonium sensitive.

ALKALINE IONS AND ACIDIFICATION BY YEAST CELLS

G. Georgiou and A. Kotyk

Mo-81

Institute of Physiology, Czech Academy of Sciences, 142 20 Prague 4, Czech Republic

Although the generally recognized acidification mechanism used by various yeast species, the plasma membrane H^+ -adenosinetriphosphatase, is not directly activated by alkaline ions, univalent cations do increase powerfully the extent of acidification brought about by addition of D-glucose, D-fructose, D-mannose, but much less by addition of D-galactose, maltose, trehalose, and not at all by ethanol, even after growth on that substrate. The decreasing order of cation effect was the following: Tl^+ , K^+ , Rb^+ , Cs^+ , (Na^+, Li^+) and was the same in the most extensively examined *Saccharomyces cerevisiae* and in the genetically best explored *Schizosaccharomyces pombe*. In the strictly aerobic *Lodderomyces elongisporus* the effect of the ions was insignificant. It appears that the ions require the presence of energy reserves, such as are generated by the glycolytic degradation of sugars. The highest acidification rate observed after addition of 20 mM KCl in *S. cerevisiae* was more than 35 nmol H^+ per min per mg dry wt.

In cells not responding pronouncedly by acidification to the addition of sugars (e.g. galactose quite generally, maltose in some cases), univalent cations bring about a sudden acidification of about 0.1 - 0.3 nmol H^+ per mg dry wt., apparently some type of exchange of surface H^+ charges for the ion added - this acidification was virtually the same after K^+ as after Na^+ and, in fact as on adding these ions to cells without any metabolizable substrate.

CORTICOSTEROIDS AND REGULATION OF K^+ -NPPASE IN RAT INTESTINAL AND RENAL EPITHELIUM DURING DEVELOPMENT

Z. Žemanová and J. Pácha

Mo-83

Institute of Physiology, Academy of Sciences of Czech Republic, Prague, Czech Republic

Corticosteroids markedly alter ion transport in target epithelia. The postnatal development of Na^+,K^+ -ATPase, the biochemical equivalent of Na-pump was studied histochemically utilizing the K^+ -NPPase activity or Na,K -ATPase. The histochemical procedure was carried out, with a few modifications, as described by Kobayashi et al. and the suitability of this method for detection of the possible effect of corticosteroids was demonstrated by the stimulation of K^+ -NPPase by dexamethazone (DXM) in adult animals. The role of adrenal steroids in the development was studied in suckling and weaning control and adrenalectomized (ADX) rats. ADX decreased the enzyme activity both in distal colon and kidney. There was not any gradient in the enzyme profile along the axis of crypt-colonic surface and ADX did not change the distribution. The staining was only partially inhibited by ouabain but the inhibition in ADX animals was always significantly less than in controls.

CORRELATION BETWEEN Mg-ATPASE ACTIVITY AND AMINOPHOSPHOLIPID TRANSLOCATION IN RED CELL MEMBRANES

Zs. Beleznay^a, A. Zachowski^b, P. F. Devaux^b and P. Ott^a

^aInstitut für Biochemie und Molecularbiologie, Bühlstrasse 28, CH-3012 Bern, Switzerland, and ^bInstitut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris, France

The phospholipids of the erythrocyte membrane are distributed asymmetrically over the two membrane leaflets; aminophospholipids are predominantly located at the cytoplasmic side and the choline-containing ones at the extracellular half of the bilayer. More recently an Mg-ATP-dependent aminophospholipid translocase has been described and postulated to be responsible for the asymmetrical disposition of those lipids. The stoichiometry between aminophospholipid translocation and ATP consumption is approx. one. This close correlation supported the notion that a Mg-ATPase participates in the ATP-driven aminophospholipid translocation. In red cell membranes two Mg-ATPase activities have been described.

In the present study, suramin inhibited Mg-ATPase activity in a biphasic manner where the more sensitive component (IC_{50} 4.8 μM) represented 40-60 % of the total activity and the less sensitive component was responsible for the remaining activity. IC_{50} for suramin of this less sensitive part of Mg-ATPase activity was ATP-dependent. When ATP concentrations were varied from 80 μM to 1.4 mM, IC_{50} increased from 60 μM to 2.9 mM. These changes reflect the competitive manner of inhibition by suramin exclusively for the low-sensitivity Mg-ATPase activity. The initial velocity of spin-labelled phosphatidylserine translocation was decreased by 70 % at low ATP concentration (300 μM) in ghosts that contained 200 μM suramin. In ghosts resealed in the presence of 54 μM suramin, the aminophospholipid translocation was inhibited competitively with respect to ATP. These data suggest that the fraction of Mg-ATPase activity that is less sensitive to suramin is the one involved in aminophospholipid translocase activity in red cell membranes.

THE EFFECT OF FREE OXYGEN RADICALS ON BRAIN ENDOPLASMIC RETICULUM CALCIUM TRANSPORT AND MEMBRANE FLUIDITY

P. Račay, P. Kaplán, G. Bezšková, J. Lehotský and V. Mézešová

Comenius University, Jessenius Medical School, Department of Biochemistry, Malá Hora 4, 036 01 Martin, Slovakia

The in vitro effect of free oxygen radicals on ATP dependent Ca uptake, Ca-ATPase and membrane fluidity was studied in rabbit brain endoplasmic reticulum (ER). Isolated microsomes were incubated with systems generating hydroxyl (Fe^{2+} -EDTA+ H_2O_2), alkoxy (Fe^{2+} -EDTA) and peroxy radicals or with H_2O_2 for 30 min. Marked decrease in Ca uptake was observed in ER samples incubated with hydroxyl, alkoxy and peroxy radicals but not with H_2O_2 . Membrane fluidity was also significantly decreased as detected by fluorescence polarization studies using probe 1,6-diphenyl-1,3,5-hexatriene. However, decrease in V_{max} for Ca-ATPase activity (without significant changes in the affinity for Ca^{2+}) was observed only after prolonged treatment (more than 10 h at 0 °C) of ER with free oxygen radicals. Changes observed in Ca uptake, Ca-ATPase and membrane fluidity were prevented by the inclusion of chain-breaking scavengers (butylated hydroxytoluene, α -tocopherol and stobadine) in the incubating medium. The results obtained are consistent with the view that altered physical state of membrane lipids, subsequent to the lipid peroxidation, plays an important role in the impairment of ER Ca-transport.

Mo-85 THE KINETIC PARAMETERS OF SYNAPTOSOMAL Na,K-ATPase DURING BRAIN ISCHEMIA AND ISCHEMIA FOLLOWED BY REPERFUSION IN MONGOLIAN GERBILS

M. Váchová, S. Macháč, J. Lehotský and V. Mézešová

Comenius University, Jessenius Medical School, Department of Biochemistry, Malá Hora 4, 036 01 Martin, Slovakia

Lack of oxygen during ischemia leads to decreased ATP level and to the failure of ATP driven ion transport systems. Na,K-ATPase activity is significantly affected during cerebral ischemia, the kinetic basis of these changes are not yet clear. Therefore we studied the kinetic parameters of synaptosomal Na,K-ATPase during brain ischemia and ischemia followed by reperfusion. Ischemia was produced by bilateral occlusion of common carotid arteries. A synaptosomal fraction was obtained by both differential centrifugation of homogenate and centrifugation at a discontinuous sucrose gradient. We found that the affinity of Na,K-ATPase for K⁺ significantly decreases during reperfusion (K_a increases by 26%) and affinity of the enzyme for Na⁺ increases (K_a decreases by 37%). Synaptosomal Na,K-ATPase exhibits two components of ATP dependence: high affinity and low affinity sites. The high affinity site had an apparent Km in ATP concentration ranging from 2 to 50 μmol/l. Low affinity site Km increases during both ischemia and reperfusion. We suggest that ischemia influences mainly dephosphorylation step of the ATPase reaction cycle. This is likely caused by an alteration of protein and/or lipid surroundings subsequent to ischemia.

Mo-87 ROLE OF INTRACELLULAR Ca²⁺ IN INHIBITION OF RESPIRATION OF EHRLICH ASCITES TUMOR CELLS BY CARBOHYDRATES

V.V. Teplova,^a K. Bogucka,^b Yu.V. Evtodienko^a and L. Wojtczak^b

^aInstitute of Biological Physics, Russian Academy of Sciences, Pushchino, Moscow Region, Russian Federation and ^bNencki Institute of Experimental Biology, Warsaw, Poland

One of the characteristic features of tumor cells is the inhibition of their respiration by glucose (Crabtree effect). The following results strongly suggest that the Crabtree effect is mediated by an increase of cytoplasmic Ca²⁺ concentration.

As shown previously [Teplova et al. (1993) Biochem. Biophys. Res. Commun. 196, 1148-1154; Czyż et al. (1993) Acta Biochim. Polon. 40, 539-544], cytoplasmic Ca²⁺ concentration in Ehrlich ascites cells is increased above 0.4 μM by addition of glucose or 2-deoxyglucose to the incubation medium. Now, we have demonstrated that isolated Ehrlich ascites mitochondria preloaded with Ca²⁺ exhibit distortion of energy-coupling processes: depression of State 3 respiration, lowering of the rate of ATP synthesis and insensitivity of the membrane potential to the addition of ADP. In digitonin-permeabilized cells these symptoms are observed when the external Ca²⁺ concentration is 0.4 μM or higher. Moreover, preincubation of cells with glucose or deoxyglucose results that, after subsequent permeabilization with digitonin, the coupled respiration becomes inhibited already at 0.3 μM Ca²⁺. From all these results it can be concluded that glucose and deoxyglucose elicit in Ehrlich ascites cells a sequence of events in which the increased concentration of cytoplasmic Ca²⁺ promotes excessive accumulation of Ca in mitochondria, and this, in turn, inhibits energy-coupling reactions.

Mo-86 PROGRESS IN STUDIES ON THE Na⁺ CYCLE
V. P. Skulachev
Belozerski Inst. Phys.-Chem. Biol.,
Moscow State Univ. Moscow, Russia

It has been postulated that the Na⁺ cycle substitutes for the H⁺ cycle at low d/uH and high [Na⁺] out. In agreement with this hypothesis, it was found that in *E. coli* and *Bac. FTU* Na⁺-motive respiration is induced by protonophores or high pH, and in *Bac. FTU* by low cyanide concentrations inhibiting the H⁺-motive respiration (our group). In *Ent. hirae*, induction of Na⁺-ATPase occurs in the presence of protonophores at high pH or in H⁺-ATPase deficient strains (Kakinuma's group). In thermophilic *Cl. fervidus* Na⁺-ATPase and Na⁺ metabolite symporters compose the Na⁺-cycle. In this case, the use of Na⁺ is due to the fact that membrane permeability is much higher for H⁺ than for Na⁺ at high temperature (Koning's group). In some cases induction of Na⁺ cycle requires synthesis of enzymes (the Na⁺-motive cyt d in *E. coli* or cyt o in *Bac. FTU*) or their modification (*E. coli* F₀F₁-type ATPase). The protonophore-induced cyt d synthesis requires Arc proteins (our group). The *E. coli* H⁺ motive cyt o is decoupled from H⁺ pumping at high pH (Wikstrom's group). Low d/uH is hardly the only reason to use the Na⁺ cycle. In *P. modestum* the energetics is based on Na⁺-motive decarboxylation under any conditions (Dimroth's group). In some methanogens, one and the same methanogenesis-linked chain of reactions includes both Na⁺- and H⁺-coupled steps. The role of this complicated energy pattern will be discussed.

Mo-88 p-NTV IS A VITAL DYE OF RESPIRATORY ENSEMBLES IN MITOCHONDRIA AND BACTERIA
A.Belyakovich

ITEB, Pushchino, Moscow region,
142292, Russia

p-NTV as a vital dye of mitochondria and bacteria, new construction of cell and dark-field light microscopy are three components of new technique to study respiratory ensembles in mitochondria and bacteria (1). The technique allows to count all mitochondria in a cell, to observe behavior of individual mitochondria, to study appearance, functioning and disappearance of respiratory blots (space of membrane where respiratory ensembles are located) in bacterium.

New results. Different types of bacteria show different kinds of respiratory blots. New blot appears de novo. It can appear both before and after cell division. In vitro mitochondria convert into microorganisms with only mitochondrial DNA. In some natural bacteria were observed both high molecular and mitochondrial DNA. On the base of all results obtained (1) it was developed the hypothesis about origination of prokaryotes from eukaryotes (2).

1. A.Belyakovich. Study of mitochondria and bacteria using tetrazolium salt p-NTV. Pushchino, 1990, 233 pp.

2. A.Belyakovich. Hypothesis about origination of prokaryotes from eukaryotes. Moscow, Hypothesis, 1993, N2 (in press).

RELATIONSHIP BETWEEN BACTERIAL CELL
INTEGRITY AND RESPIRATORY CHAIN ACTIVITY:
A FLUORESCENCE ANISOTROPY STUDY

Mo-89

T.V. Votyakova, A.S. Kaprelyans

A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, 117071 Moscow, Russia

The fluorescence anisotropy (r) of intracellular NADH was used as a natural fluorescent probe to monitor intracellular structure and cell integrity of *Micrococcus luteus*. In freshly grown cells this parameter was equal to 0.18 - 0.21, indicating a high degree of immobilization of intracellular NADH (in water $r = 0.08$). The increase of medium osmolarity (0 - 2.5 M xylitol) caused a subtle increase in the parameter r . In contrast to cells, protoplasts were sensitive to medium osmolarity: r value decreased from 0.21 to 0.15 when the xylitol concentration was varied from 1.0 to 0.7 M. Below 0.7 M r reached the minimum of 0.09 - 0.1 due to protoplast lysis. Thus significant portion of NADN in intact cell is reversibly bound. The equilibrium can be shifted with protoplast swelling that is reflected on corresponding changes in r value. When cells were subjected to gentle lysis with lysozyme in the presence of saturating concentrations of respiratory substrates, the transient stimulation of respiratory rate was observed. The following decrease in respiratory chain activity correlated with r value reduction. Bacterial cells subsequently treated with glutaraldehyde and lysozyme demonstrated preservation of high respiratory activity for along time. It is concluded that being treated with lysozyme, the respiratory chain of intact *M. luteus* cells exist *in situ* in a state characterized by a maximal activity, until cell integrity is disturbed.

CYTOTOXIC ALKALOID SANGUINARIN AS INHIBITOR OF ENERGY TRANSDUCTION IN MITOCHONDRIA AND OF LYSOSOMAL HYDROLASES

Mo-91

A.Bulychev, T.Belyaeva,
E.Leontyeva, M.Faddejeva

Laboratory of Biochemical Cytology and Cytochemistry, Institute of Cytology of the RAN, St.Petersburg, Russia.

During investigations on intracellular targets for cytotoxic plant benzophenanthridine alkaloid sanguinarine, (S), the accumulation of (S) into mitochondria and lysosomes of L-cells has been determined by means of fluorescent microscopy technique using LUMAM-III (LOMO).

In isolated rat liver mitochondria (S) involved maximum 22-fold increase of latent ATPase activity at 5×10^{-6} M of (S) and inhibited this activity after further raising of (S) concentration. (S) evoked similar effects on the rate of mitochondrial oxygen consumption. (S) lowered also the respiration control index and ADP/O relation. All the facts indicated the disturbances of energy transduction processes in mitochondria by (S). The organic cations of (S) have been proposed to neutralize the negative charges near the external side of energized internal mitochondrial membranes, which were indispensable for ATP synthesis.

Incubation of L-cells in a medium containing 0.2 mM of (S), caused the inhibition of near 50% for activities of lysosomal hydrolases, ATPase lysosomes being essential for these effects.

Mo-90 CITRATE EFFLUX AND PLASMA MEMBRANE PROTON ATPASE IN *PENICILLIUM SIMPLICISSIMUM* ARE STIMULATED BY A BUFFER

W. Burgstaller, A. Zanella and F. Schinner

Institute of Microbiology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria

A high extracellular buffering capacity - provided by 1 M MES-NaOH/pH 6 or 1 M HEPES-NaOH/pH 7.5 - increases the long-term (10 d) excretion of citrate by the filamentous fungus *Penicillium simplicissimum* more than 50-fold. Even more citrate is excreted if zinc oxide is used as a buffer ($ZnO + 2H^+ \rightarrow Zn^{++} + H_2O$). All three proton "sinks" influence the pmf between the two bulk phases, the H^+ -concentration near the outer surface of the plasma membrane and the intracellular pH. Each of these parameters has been described to affect the activity of the plasma membrane H^+ -ATPase and the plasma membrane potential gradient.

At least in the presence of ZnO , citrate efflux contributes to charge compensation for outward pumping of protons by the H^+ -ATPase. This was concluded because in the presence of the freely permeable cation tetraphenylphosphonium (TPP) citrate efflux was reduced (H^+ -excretion and O_2 -consumption were not reduced by TPP, nor was intracellular citrate). Unlike TPP, buffer-stimulated citrate efflux was not reduced by 150 mM potassium.

It was further found that the Mg-dependent, azide-, molybdate- and nitrate-insensitive, but ortho-vanadate-sensitive ATP-hydrolyzing activity measured at pH 6 in an enriched plasma membrane fraction was increased if the cells were exposed to 0.5 M HEPES-NaOH/pH 7.5 for 10 minutes prior to cell breakage. This stimulation was twice as large as the activation brought about by glucose in the absence of a buffer.

These results support the view that changes in the activity of the H^+ -ATPase and in the membrane potential - two tightly linked parameters - are part of the metabolic reactions leading to buffer-stimulated citrate efflux.

Mo-92 THE REACTION OF CYTOCHROME OXIDASE WITH CYANIDE AND CRYOPROTECTORS

HILDA JALČOVÁ,^a PAVOL JALČ ^b

^a INSTITUTE OF EXPERIMENTAL PHYSICS OF SLOVAK ACADEMY OF SCIENCES, BIOPHYSICAL LABORATORY, 047 53 KOŠICE, WATSONOVÁ 47.

^b INSTITUTE OF NEUROBIOLOGY SLOVAK ACADEMY OF SCIENCES, 04 001 ŠROBÁROVÁ 46, KOŠICE, SLOVAKIA.

CYTOCHROME OXIDASE IS A HEMOPROTEIN IN EUKARYOTES. LOCATED IN THE INNER MITOCHONDRIAL MEMBRANE WHERE IT FORMS THE LAST COMPONENT OF THE ELECTRON TRANSFER CHAIN.

CYTOCHROME C OXIDASE WAS ISOLATED FROM BEEF HEART BY THE METHOD OF YONETANI: THE PREPARATIONS CONTAINING $7 - 11 \cdot 10^{-9}$ MOL HEME a / mg OF PROTEIN WERE USED FOR THE ENTIRE INVESTIGATION.

THE DEPENDENCES OF THE PRESENT OF $K_4Fe(CN)_6$ ON CONCENTRATION OF CRYOPROTECTORS GLYCEROL, 1 - 2 PROPANEDIOL AND DIMETHYLSULPHOXIDE IN CONCENTRATIONS UP TO 30 - 50 % BY VOLUME WERE USED.

MO - 93 MEMBRANE MITOCHONDRIAL FUNCTIONALITY AND CARDIOLIPIN ORGANIZATION OF ADRIAMYCIN RESISTANT K562 CELLS.
M. Denis-Gay, J.M. Petit, M.H. Ratinaud
Institut de Biotechnologie, Faculté des Sciences, 87060 LIMOGES Cédex - FRANCE.

The incorporation of Rhodamine 123 (Rh123), a mitochondrial potential dependent probe, in adriamycin (ADR) resistant K562 cells (K562/R) seems not directly drive by the proton-motive force. Indeed, the Rh123 uptake in K562/R cells was slight and occurred quasi-independently of temperature, in contrast to the sensitive cell line (K562/S). After washing, the cell dye retention was lower in K562/R cells (<20%) than in K562/S cells (86%). The retention was not completely restored by addition of verapamil which blocks the efflux of positively-charged compounds. Moreover, microscopic observations of K562/S cells after Rh123 staining showed a bright fluorescence specifically located in chondriome, while in K562/R cells a weak fluorescence was observed in cytoplasm. Whatever, the cell physiologic state of K562/R cells (logarithmic and stationary growth phases), the results obtained were similar using Rh123. Biochemical investigations were carried out in order to understand precedent results. A decrease in oxygen consumption and respiratory chain enzyme activities were observed in three quiescent cell lines in comparison to cycling cells. A dramatic difference in cytochrome c oxidase activity between the two physiologic states was only observed in K562/R cells.

Otherwise cardiolipin (CL) was studied, owing to its involvement in enzyme activities of the inner mitochondrial membrane. The amount of this phospholipid in chondriome and its transverse distribution in the inner membrane were performed with 10-N Nonyl Acridine Orange, a cardiolipin probe. In ADR resistant cells, the cardiolipin content increased by 26%, comparatively to sensitive cells. On the other hand, in this last cell line about 50% of total CL was located on the cytoplasmic face of inner membrane, whereas the distribution was greatly in favour of the outer leaflet (81%) in K562/R cells.

MO - 95

THE INFLUENCE OF THE ALTERNATIVE OXIDASE ON XYLOSE FERMENTATION WITH THE YEAST *PICHIA STIPITIS*.

H. Jeppsson and B. Hahn-Hägerdal
Applied Microbiology, Lund Institute of Technology/University of Lund, P.O. Box 124, S-221 00 Lund, SWEDEN

Xylose fermenting yeasts require a critical level of oxygenation for maximum ethanol yield and ethanol productivity during xylose fermentation. The oxygen requirement of the xylose fermenting yeasts has been suggested to relieve the redox imbalance caused by the two first steps in the xylose metabolism. Xylose is first reduced to xylitol by a NADPH-linked xylose reductase (XR) followed by oxidation of xylitol to xylulose by a NAD⁺-linked xylitol dehydrogenase (XDH). A redox imbalance is indicated by xylitol excretion during xylose fermentation. The XR of *Pichia stipitis* and of *Pachysolen tannophilus* can use either NADPH or NADH as cofactor and thereby the redox imbalance caused by the two first steps in the xylose metabolism should be circumvented. Despite this dual cofactor use for XR, *P. tannophilus* excretes xylitol during xylose fermentation. *P. stipitis* on the other hand produces very little xylitol suggesting that a redox imbalance does not exist in this organism.

The influence of the respiratory inhibitors cyanide and salicyl hydroxamic acid (SHAM) on xylose fermenting yeasts (*P. stipitis*, *P. tannophilus* and *Candida utilis*) and on *Saccharomyces cerevisiae* showed that *P. stipitis* has a mitochondrial cyanide resistant SHAM sensitive alternative oxidase. The alternative oxidase was found in both glucose and xylose utilising cells of *P. stipitis*. *P. stipitis* excreted polyols including xylitol during xylose fermentation when the alternative oxidase was inhibited by SHAM. This suggests that NAD⁺ required for the oxidation of polyols is regenerated by the alternative oxidase.

MO - 94 NITRIC OXIDE INDUCES POST-TRANSLATIONAL MODIFICATIONS OF ERYTHROCYTE BAND 3 AND G3PDH
C. Mallozzi, A.M.M. Di Stasi, G. Scorzà, M. Minetti
Cell Biology, Istituto Superiore di Sanità, Rome (Italy)

Nitric oxide (NO) is a major biological messenger molecule accounting for endothelium-derived relaxing factor activity in blood vessels and the tumoricidal and bactericidal actions of macrophages as well as serving a neurotransmitter-like role in the brain. NO exerts some of its actions by stimulating guanylyl cyclase, which is not the only physiologic target of NO, however. It has been previously demonstrated that NO can enhance NAD-covalent modification of a 37-KDa cytosolic protein, identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH). In the present study we suggest that erythrocytes represent another physiologic target of NO by regulating its removal from plasma and its detoxification through the glycolytic cycle. Intact human red blood cells (RBC) in the presence of a physiologic NO donor (S-nitroso thiol of serum albumin) produced stoichiometrically methemoglobin (metHb) after 10 min at 37°C. In the cells, metHb was efficiently reduced to hemoglobin (HbO₂) by plasma, glucose or NADH, but not by NADPH or riboflavin. The conversion of metHb back to its functional form depends on the generation of NADH during dehydrogenation of G3P by G3PDH. We found that human RBC ghosts exposed to different NO donors in the presence of [³²P] NAD showed an increased radioactivity incorporation in the band corresponding to G3PDH. The labeling was almost completely inhibited by HbO₂, but not by Catalase and/or SOD. Moreover, NO treatment induced tyrosine phosphorylation of Band 3 in intact RBC. Tyrosine phosphorylation of Band 3 may prevent the binding of G3PDH thus allowing its translocation. We hypothesize that the transport of NO across the RBC membrane induces post-translational modifications of Band 3 and G3PDH, and, as consequence, activation of glycolysis to maintain the HbO₂ in its reduced functional form.

BACTERIORHODOPSIN PHOTOCYCLE IN CHARGE PULSE

MO - 96 **Valeriu Cismasiu, Dan Mihailescu,**
Univ. of Bucharest, Fac. of Biology,
Bacteriorhodopsin (BR), the retinal-protein complex in the purple membrane (PM) of *Halobacterium halobium*, functions as a light-driven proton pump. Upon light absorption, light-adapted BR undergoes a reaction cycle which, after the isomerization of the retinal, proceeds through a succession of thermal steps of the protonated and unprotonated intermediate forms. Disagreement still exists concerning the exact sequence of the photochemical cycle and even the number of intermediate forms. Photoelectric studies have been carried out on the PM attached to BLM, but the difference from these is that here we applied short-time electric fields to compound membrane. The sequence of proton release and uptake and the net protonation state of the intermediates relative to steady-state BR are different in the low and high pH pathways: at pH < 5 the uptake occurs before the release, while at pH > 8 the release precedes the uptake. The reversible reactions are associated with elementary charge displacements. The kinetics of these steps can be perturbed by applying charge pulses in a definite moment, differently for the two pH pathways. This perturbation results in the change of the life times, depending on the polarity and the magnitude of the pulses. The interpretation of these changes depends on the kinetic model (branched or not) adopted for the cycle. The rate of pumping may change when the different charge pulse relaxed in the time of occurring the only reversal steps.

EFFECTS OF INHIBITORS OF ENERGY METABOLISM ON STRUCTURE AND FUNCTION OF CELLS IN CULTURE

Mo-97 I. Polyakova, M. Leikina, D. Zorov

Department of Cytology and Histology, Biological Faculty, Moscow State University, Russia

We have studied the effects of inhibitors of oxidative phosphorylation with different action mechanism and hypoxia on the mitochondrion in cultured cells.

It has been established that inhibitors of electron transport (amytal, rotenone, antimycin A), uncouplers (2,4-dinitrophenol), inhibitors ATP-synthetase (oligomycin, diciclohexylcarbodiimid) and hypoxia induced morphological and functional changes in cultivated cells and especially in mitochondrion:

- the rate of oxygen consumption decreased (cell respiration was measured by polarographic method);

- the membrane potential of mitochondria decreased and mitochondrial reticulum disintegrated to single small mitochondria (luminescent-microscopic study the cells by vital staining with rhodamine 123);

- cell ultrastructure changed: polysomes were desintegrated into single ribosomes, mitochondria got condensed or swelling conformation, cistern of rough endoplasmic reticulum and perinuclear space swelled;

- intracellular pH decreased.

So, degree and dynamics of described changes are specific for the each effect.

Morphological and functional changes can be reversible after effect removal.

STRUCTURE-FUNCTIONAL INTERACTION OF FATTY AND DICARBOXYLIC ACIDS OXIDATION IN UNCOUPLED MITOCHONDRIA

N.I. Fedotcheva, N.V. Svyatukhina, Y.Y. Scarga, A.G. Fogel and M.N. Kondrashova

Institute of Theoretical and Experimental Biophysics, Russian Acad. Sci., Pushchino, Russia

The structure-functional interaction of fatty and dicarboxylic acids oxidation in uncoupled liver and brown fat mitochondria was studied. Oxidation of succinate is shown to be spontaneously inhibited due to higher oxalacetate formation than its utilization. The inhibition is increased by malate and is abolished by acetyl- or palmitoyl-carnitine. Succinate activated palmitoyl-carnitine oxidation in uncoupled mitochondria more extensively than malate. The rate of simultaneous oxidation of succinate and fatty acid was higher than separate oxidation rates. This assumes the existence of multienzyme complex oxidizing these substrates. The preincubation of mitochondria with antibodies to malate dehydrogenase blocked completely both the endogenous respiration and malate oxidation but having no effect on succinate oxidation in the presence of rotenone. The interaction of antibodies with malate dehydrogenase was considerably higher in brown fat than in liver mitochondria. These data evidence that the increase amount of malate dehydrogenase serves as one more mechanism which provides high rate in brown fat mitochondria.

UNITARY THEORY OF CHEMIOSMOTIC COUPLING FOR CELL PLASMA MEMBRANES

Mo-98

K.B. Aslanidi

Laboratory for Cellular Biophysics, Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Russia.

The application of P.Mitchell chemiosmotic concept for plasma membranes is very important for understanding of the matter turnover in living cell realized by energy fluxes through self-organized system.

We elaborated the mathematical model, that couples the rate of ATP hydrolysis by an ionic pump with the plasma membrane potential, ionic fluxes, intracellular ion concentrations and cell volume. But the analysis of various properties of single cell membrane is difficult and moreover, not enough for elaboration of an unitary theory of chemiosmotic coupling. However the multicellular system with intercellular permeable junctions (PJ) gives possibility to separate mechanisms of active and passive ion transport to different cells. We demonstrated that (i)ionic currents through PJ couple transport processes in adjacent cells and electric currents determine the energetic cooperation between cells, (ii)an uncompensated transfer of one positive charge via PJ is equivalent to transfer of the energy liberated by one ATP molecule hydrolysis in opposite direction. Connection between transport processes, membrane electrogenesis and energetic metabolism was found in some evolutionary distant organisms

The idea of intercellular cooperation via PJ gives possibility to understand a great number of effects in the phenomenon of tissue self-organization. Association of this idea with the bicoenergetic concept allows to predict new effects in some areas of fundamental researches in medicine and biotechnology.

THE PROTON PUMP OF HEME-COPPER OXIDASES

S. Papa

Mo-100 Institute of Medical Biochemistry and Chemistry, University of Bari, Italy.

Proton pumping heme-copper oxidases represent the terminal, energy-transfer enzymes of respiratory chains in prokaryotes and eukaryotes. Much progress is being made in the elucidation of the conversion of redox energy into transmembrane protonmotive force by oxidases, based on mutational analysis of prokaryotic enzymes and sophisticated spectral analysis of reaction intermediates. The Cu_b-heme a₃ (or heme o) binuclear center, associated with the largest subunit I of cytochrome c and quinol oxidases, is directly involved in the coupling between dioxygen reduction and proton pumping. The role of the other subunits is less clear. The following aspect will be covered in this paper: i) the efficiency of coupling in the mitochondrial aa₃ cytochrome c oxidase. In particular the effect of respiratory rate and protonmotive force on the H⁺/e⁻ stoichiometry (Papa et al. FEBS Lett. 1991, 288, 183) and the role of subunit IV; ii) mutational analysis of the aa₃ quinol oxidase of *Bacillus subtilis* addressed to the role of subunit III, subunit IV and specific residues in subunit I (G. Villani et al. EBEC, 8, 1994, manuscript in preparation); iii) possible models of the protonmotive catalytic cycle at the binuclear center. The observations available suggest that H⁺/e⁻ coupling is based on combination of protonmotive redox catalysis at the binuclear center and cooperative proton transfer in the protein.

Mo - 101 ROLE OF MITOCHONDRIAL ENERGY METABOLISM IN THE REGULATION OF LIVER REGENERATION
F. Guerrieri
Institute of Medical Biochemistry and Chemistry and C.S.M.M.E., CNR, Bari, Italy

During liver regeneration, an adequate supply of cellular energy for biosynthesis of cellular components is required. Under normal conditions, mitochondrial oxidative phosphorylation is the main process to cover the energy demand of the cell. Mitochondrial energy metabolism during the various phases of liver regeneration was studied in three different conditions in which the rate of rat liver regeneration was influenced by: i) age of the rats; ii) injection of a synthetic pentapeptide inhibitor of liver regeneration; iii) reduced thyroid function by injection of propylthiouracil (PTU). During early liver regeneration a lag phase in the growth of the liver is observed in which a decrease of the rate of ATP synthesis and of immunodetection of ATP synthase subunits occurs. This lag phase is longer in aged and PTU treated rats. After the lag phase the recovery of the mass is accompanied by recovery of activity and amount of immunodetected ATP synthase subunits is observed. It can be concluded that cell growth in the regeneration process is associated with a coordinated assembly of the F₀F₁ ATP synthase, this providing the main source of ATP. During the early phase alterations of the enzyme occur which could be related to alteration in the turnover of the protein (synthesis end proteolysis).

Mo - 103 GENES OF ENERGY BALANCE: MODULATION IN TRANSGENIC MICE, *Leslie P. Kozak* The Jackson Laboratory, Bar Harbor, Maine, USA.

An efficient metabolism, essential for survival in a foraging, physically-active primitive culture, has been postulated to lead to obesity and diabetes in modern man. We seek to understand the genetic basis of energy efficiency with transgenic mouse models and mutations which inactivate the relevant genes. Two genetic models have been developed: 1) Over-expression of the cytoplasmic NAD-linked glycerol-3-phosphate dehydrogenase generates a futile ATPase which burns off energy and results in a depression of non-shivering thermogenesis and a reduction in white fat lipid stores. These effects are observed in normal transgenic mice and in transgenic mice also homozygous for the diabetes (*db*) gene. The results indicate that modulations in the basic pathways of carbohydrate and lipid metabolism can have profound effects on energy efficiency. 2) The second model alters energy efficiency by modulating expression of the mitochondrial uncoupling protein, a key step in the pathway of non-shivering thermogenesis. A transgene has been produced in which the brown fat has been ablated by a transgene which has the *Ucp* regulatory region driving expression of the diphtheria toxin gene. These mice, which have a major reduction of brown fat, are obese suggesting that the loss of brown fat increases the energy efficiency in these animals. Verification for such a major role for brown fat in energy balance and obesity in mammals is being sought by inactivating the *Ucp* gene by homologous recombination in embryonic stem cells

Mo - 102 PHOSPHOENOLPYRUVATE TRANSPORT IN RAT AND RABBIT KIDNEY MITOCHONDRIA
A. Atlante*, P. Pierro^b, Z. Drahota^c, S. Passarella^d and E. Quagliariello^b

*Centro di Studio sui Mitocondri e Metabolismo Energetico CNR, Bari, Italy; ^bDipartimento di Biochimica e Biologia Molecolare, Università di Bari, Italy; Institute of Physiology, CzAcadSci, Praha, Czech Republic; ^cDipartimento di Scienze Animali, Vegetali e dell' Ambiente, Università del Molise, Campobasso, Italy

Mitochondria can only function if there is a continual interchange of metabolites and end products with the cell cytosol, thus about twenty different translocators have been identified in mammalian mitochondria. Nevertheless further investigation seems necessary both to ascertain the occurrence of such translocators in mitochondria from different tissue in various animal species and to identify the existence of special carriers involved in the specific metabolic reactions that has not yet been sufficiently identified. The knowledge about the permeability properties of isolated rabbit kidney mitochondria is very poor, in particular even if the tricarboxylate carrier proved to mediate both a citrate/PEP and malate/PEP exchanges in rat liver, the mechanism of phosphoenolpyruvate (PEP) transport across the mitochondrial membrane remains to be established. PEP metabolism occurs essentially by means of phosphoenolpyruvate carboxykinase whose activity in the rat is predominantly found in cytosol, whereas significant amounts are found in both cytosol and mitochondria in the rabbit. In this work comparison was made between rat and rabbit kidney mitochondria with respect to PEP transport. Evidence will be given of the existence of new mitochondrial carriers which mediates PEP uptake mitochondria in exchange with its metabolic products and viceversa.

Finally the role of PEP translocators in its metabolism, in rat and rabbit kidney, will be discussed.

Mo - 104 COLD ACCLIMATION OF HAMSTERS CHANGES THE LEVELS OF G PROTEINS IN BROWN ADIPOSE TISSUE PLASMA MEMBRANES

J. Novotny, P. Kvapil and L.A. Ransnas
Wallenberg Laboratory for Cardiovascular Research, Gothenburg University, S-41345 Gothenburg, Sweden

The effect of cold acclimation on the distribution as well as functional characteristics of heterotrimeric G proteins was studied in brown adipose tissue (BAT) of golden hamsters. The levels of G proteins in plasma membranes prepared from BAT of control and cold-acclimated hamsters were determined by quantitative immunoblot analysis and competitive ELISA. Cold acclimation decreased significantly the total content of G_{αα} (by 16%), G_{βγ} (by 28%) as well as β subunits (by 31%) of G proteins in BAT plasma membranes. Interestingly, the reduction in G_{αα} content was solely due to a large reduction in the content of the short (45 kDa) isoform of G_{αα} (by 59%), while the level of the long (52 kDa) isoform of G_{αα} remained unchanged. Quantitative alterations induced by cold acclimation in BAT plasma membranes associated G protein network were accompanied by changed functional characteristics of this network. While no change was found in forskolin-sensitive adenylyl cyclase (AC) activity, a decrease of AlF₄-sensitive AC activity (by 29%) as well as cyc^c-reconstitutive AC activity (by 35%) was detected in BAT plasma membranes of cold-acclimated hamsters. In conclusion, altered regulation of splicing of G_{αα} mRNA and/or G_{αα} forms viability in BAT during cold acclimation presents a new evidence about physiological inequality of splicing variants of G_{αα}.

B₃-ADRENERGIC RECEPTORS AND ENERGY EXPENDITURE

Mo - 105 D.K. Kreutter, D.M. Hargrove, J. Chin and R.W. Stevenson
Pfizer Inc., Central Research Division,
Groton, CT, USA

BRL35135 and ICI198157 are selective B₃ agonists that stimulate oxygen consumption in rodents. These compounds are both full agonists at the cloned rat B₃ receptor, but only partial agonists at the human B₃ receptor. It has recently been demonstrated that the cloned human B₃ receptor is not a full length receptor. The intrinsic efficacy of BRL35135 and ICI198157 is much lower at the full length receptor than at the truncated receptor, which correlates with the poor intrinsic efficacy reported for the stimulation of lipolysis by B₃ agonists in isolated human adipocytes. Using rodent models, we examined whether skeletal muscle might be a site at which B₃ agonists act to stimulate thermogenesis, or whether their action is limited to brown fat. In isolated soleus muscle, isoproterenol, a non-selective B agonist, stimulates adenylyl cyclase and completely suppresses insulin-stimulated glycogenesis. BRL35135 also stimulates adenylyl cyclase and completely suppresses insulin action, whereas ICI198157 was without effect. The effects of isoproterenol and BRL35135 are reversed by the B₂ antagonist, ICI118551, but not by the B₁ antagonist, practolol, demonstrating that the B₂ receptor is primarily responsible for mediating the effects of B adrenergic agonists on skeletal muscle. The effect of BRL35135 is due to activation of B₂ receptors by this compound, while the lack of effect of ICI198157 is due to the more complete selectivity of this compound for the B₃ receptor. We are unable to detect B₃ receptor mRNA in rat or human skeletal muscle by either ribonuclease protection or PCR. B₃ receptor mRNA is present in human adipose tissue, and in some sites is coexpressed with uncoupling protein. These data suggest that the thermogenic effect of selective B₃ agonists in man is not due to skeletal muscle thermogenesis, but analogous to rat, may be due to enhanced expression of uncoupling protein in brown adipose tissue.

Mo - 107 NEUROPEPTIDES KYOTORPHIN AND NEKYOTORPHIN AS REGULATORS OF PROLIFERATION OF NON-NEURONAL CELLS : TEST ON BROWN PREADIPOCYTES

G.Bronnikov, V.Golozoubova, J.Nedergaard & B.Cannon
The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, S-10691 Stockholm, Sweden

Brown adipose tissue constitutes an interesting model for studies of cell proliferation and cellular differentiation. The tissue plays a major role in producing of the heat necessary for new-borns, hibernating animals and small mammals living in the cold. At present, a number of peptides are known as potential endogenous regulators of seasonal physiological functions in hibernating animals. Using the brown fat cell culture system, we have studied the ability of kyotorphin (Tyr-Arg) and neokyotorphin (Thr-Ser-Lys-Tyr-Arg) to affect cell culture growth and to modulate the adrenergic stimulation of the cell proliferation.

In order to analyse effect of the peptides in absence of "classical" growth factors, a system for culture of brown fat cells under fully defined conditions has been developed. It was found that neokyotorphin (NKT) and kyotorphin act on cell proliferation in opposite directions. Stimulation of DNA and protein synthesis in brown preadipocytes by NKT in both serum-containing and serum-free media was obtained. In the presence of serum, the magnitude of the effect of 1 μM NKT was comparable with the effect of 1 μM norepinephrine (NE). Without serum, there was a shift in the maximal effect to lower concentrations of NKT and the peptide slightly increased the effect of NE on cell proliferation. Kyotorphin had a complex effect on cell proliferation in the presence of serum and had no effect in serum-free medium. However, 0.01-1 μM kyotorphin in either medium inhibited the cell proliferation stimulated by 1 μM NE. The action of the peptides is not mediated through cAMP, but most probably via an elevation of intracellular Ca²⁺. The effects of the peptides on the cell culture clearly show that peripheral tissue cells contain receptors for these neuropeptides. We speculate that outside of the central nervous system, these peptides have not an analgesic, but another function, such as regulation of mitogenic activity.

CELL PROLIFERATION IN BROWN ADIPOSE TISSUE

Mo - 106 Barbara Cannon, Anders Jacobsson, Tore Bengtsson, Gennady Bronnikov, Stefan Rehnmark, Håkan Thörnberg, Pertti Kuusela, Ulf Andersson, Petr Tvrdík, Josef Houšek and Jan Nedergaard, The Wenner-Gren Institute, The Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden

Already in the early sixties, Cameron and Smith demonstrated that the mitotic index of brown adipose tissue was dramatically increased by cold exposure. The mitotic activity is found in endothelial cells, interstitial mesenchymal cells and adipocyte precursors but not in the mature adipocytes. In intact animals, the physiological initiator of the increased cell proliferation is norepinephrine. The rather unusual aspect of the norepinephrine action is that the receptor involved is a β-receptor, with cyclic-AMP as the intracellular mediator. In most other cell systems, increased cyclic-AMP is rather a negative modulator of cell division. In cultured brown adipocytes, norepinephrine-stimulated DNA synthesis could be inhibited by CGP-12177 which is a β₁-antagonist but a β₃-agonist. Thus, DNA synthesis is mediated by β₁-receptors. In the young, proliferative cells β₁-mRNA is present, and β₃-mRNA does not appear until the cells commence differentiation. Cell proliferation is often associated with increase in expression of the proto-oncogene *c-myc*. In brown adipose tissue, *c-myc* has been shown to be expressed during fetal development of the tissue, but there does not seem to be a requirement for a dramatic increase in *c-myc* expression for the cold-induced increase in proliferation to commence. A mediatory role for *c-myc* when proliferation is stimulated via a cyclic-AMP-dependent pathway does not therefore seem to exist. The proto-oncogene *c-fos* is, however, markedly increased due to cold exposure. Also the involvement of the transcription factors C/EBPα and C/EBPβ in proliferation of brown adipocytes has been investigated. C/EBPα mRNA is decreased after norepinephrine stimulation in proliferatively competent cells, although it is increased by norepinephrine stimulation later. This decrease is also seen in brown adipose tissue immediately upon cold exposure of animals and is perhaps a prerequisite for the initiation of proliferation. Both α₁- and β-pathways seem to be required to bring about the decrease. There are doubtless other transcription factors whose expression is influenced by norepinephrine and, with time, their roles will hopefully be clarified.

ANALYSIS OF THE FUNCTIONAL ORGANIZATION OF THE MITOCHONDRIAL UNCOUPLING PROTEIN

Mo - 108 F. Bouillaud^a, B. Miroux^a, S. Raimbault^a, C. Levi-Meyrueis^a, I. Arechaga^b, S. Prieto^b, E. Rialb and D. Ricquier^a

^aC.N.R.S., Centre de Recherche sur l'Endocrinologie Moléculaire, Meudon, France ; ^bC.S.I.C., Centro de Investigaciones Biológicas, Madrid, Spain.

Uncoupling protein (UCP) is a 33KDa protein found in brown adipocyte mitochondria and acting as a proton short-circuit which induces uncoupling of respiration from ATP synthesis and subsequent energy dissipation as heat. Nucleotides inhibit UCP activity whereas free fatty acids are activators.

We have developed strategies dealing with the functional organization of the UCP in the membrane. A library of bacterial clones randomly expressing antigenic sequences were selected. The ability of short UCP subsequences to select antibodies was used to study the topological organization of the UCP in the membrane. The orientation of the N-terminal extremities of the first, second, third, fourth and sixth predicted α-helices was determined.

Using site-directed mutagenesis, it was demonstrated that sequential replacement by serine of cysteine residues in the UCP does not affect either its uncoupling activity or its regulation by nucleotides and fatty acids. Deletion of Phe 267, Lys 268 and Gly 269, resulted in a mutant UCP where proton leak can be activated by fatty acids but not inhibited by nucleotides.

Mo-109 INVESTIGATING BROWN ADIPOSE TISSUE WITH NUCLEAR MAGNETIC RESONANCE. A SPECTROSCOPIC STUDY

C. Zancanaro^a, R. Nanob, C. Marchioro^c, A. Sbarbatia,
A. Boicelli^b, F. Osculatia^a

^aInstitute of Human Anatomy and Histology, Verona; ^bNMR Research Lab, IRCCS S. Raffaele, Milan; ^cSpectroscopy Lab, Glaxo Research Group, Verona, Italy

Previous work from our laboratory showed that magnetic resonance imaging is able to localize brown adipose tissue (BAT) in the living animal and that the NMR signal correlates with tissue morphology and functional condition. Preliminarily to afford the *in vivo* topical NMR characterization of BAT, we investigated tissue extracts and isolated brown adipocytes by ¹H, ¹³C, and ³¹P NMR spectroscopy.

Results showed that quantitative changes in lipid composition at different ages and during cold acclimation are detected by ¹H NMR spectroscopy in Folch (chloroform fraction) extracts of BAT. ¹³C NMR spectroscopy revealed modifications in the relative proportions of unsaturated fatty acids. ¹H NMR spectroscopy of delipidated whole tissue extracts showed peaks attributable to aminoacids, lactate and glucose. Peaks assigned to lipid, glucose, glycerol, choline and ethanolamine were found in ¹H or ¹³C spectra of isolated brown adipocytes and the effect of insulin (100 μU/ml) on adipocyte glucose content demonstrated. Preliminary ³¹P spectroscopy study of BAT extracts revealed peaks assigned to a number of phospholipids and age-related modifications of the phosphatidylcholine/phosphatidylethanolamine ratio was found. Comparison with similar experiments utilizing white adipose tissue and isolated white fat cells strongly suggested that differentiation of the two tissues by means of NMR spectroscopy is in principle feasible.

These results demonstrate that NMR spectroscopy is a useful tool for BAT investigation and open a way to the study of BAT metabolism and function in the living animal by means of topical NMR spectroscopy.

Mo-111 EXPRESSION OF IL-1 α AND IL-1 RECEPTOR TYPE I IN MOUSE BROWN ADIPOSE TISSUE AND IN PRIMARY CULTURE OF BROWN FAT CELLS

L. Burýšek and J. Houštěk

Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic

At thermoneutral conditions, high steady state levels of transcripts for IL-1 α and its receptor IL-1R α were found in specialized thermogenic organ, brown adipose tissue (BAT) of adult mice, as compared with levels in lymph nodes, brain and spleen. Similarly, high content of IL-1 α protein, which was present both as the IL-1 α precursor (31 kDa) and IL-1 α mature protein (17 kDa), was found in BAT. Comparably high levels of transcripts of both genes and the content of IL-1 α protein were found in brown fat cells in culture.

Subsequently, the influence of lipopolysaccharide (LPS) (the acute fever inducer) and of cold (brown fat activator) on expression of IL-1 genes in adult mice was investigated. A pronounced decrease of IL-1 α mRNA level in BAT was observed 24h and 72h after LPS administration and 72h after exposure to cold. Likewise, LPS decreased the IL-1R α mRNA level and depressed also the expression of cold inducible genes for the BAT specific heat producing uncoupling protein and for lipoprotein lipase. It is concluded that, besides the established centrally mediated effects of IL-1 cytokines on thermogenic function of BAT, there exists also a direct peripheral interaction of IL-1 cytokines with BAT cells.

ECTOPIC EXPRESSION OF THE MITOCHONDRIAL UNCOUPLING PROTEIN GENE IN WHITE FAT

J. Kopecky^a, S. Enerback^b, G.T. Clarke^b, M. Rossmeisl^a, Z. Hodný and L.P. Kozak^b

^aInstitute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4; ^bThe Jackson Laboratory, Bar Harbor, ME 04609, USA

While mammalian white fat is specialized for storing nutritional energy in triglycerides, brown fat is equipped for energy dissipation. Thermogenesis in brown fat depends on expression of the mitochondrial uncoupling protein gene (*Ucp*). This expression is specific for brown fat where it is thought to be essential during exposure to cold and possibly as a mechanism for energy balance during overfeeding. We are interested in relationship between brown fat thermogenesis and obesity. In order to test the potential role of *Ucp*-directed thermogenesis in energy balance, we created mice that have ectopic expression of *Ucp* in white fat, by introducing a transgene which carried *Ucp* under control of the enhancer region of the aP2 gene. This enhancer will direct expression of *Ucp* to both brown fat and white fat. As predicted expression of the *Ucp* was detected in both subcutaneous and abdominal fat depots as well as in brown fat. We determined that the level of uncoupling protein in white fat depots of transgenic animals was approximately 10% of that detected in brown fat, no increase in this protein level could be detected in brown fat of transgenic animals over that observed in nontransgenic animals. This pattern of ectopic *Ucp* expression did not reduce the total body weight of the transgenic mice on the normal C57BL/6J background, but did result in about a 20% reduction in body weight when the transgene was combined with *A^w*, a gene which causes obesity in mice. In both C57BL/6J and *A^w* mice a redistribution of fat depots occurred in which the subcutaneous fat was reduced several fold, while gonadal fat depots were correspondingly increased. Experiments on the effect of the transgene expression on the development of obesity induced by high-fat diet in C57BL/6J mice and on mitochondria in white fat will be described.

Mo-112 PROTEIN KINASE C₂ IS INVOLVED IN REGULATION OF Ca²⁺ CHANNELS IN PLASMALEMMA OF NITELLA SYNCARPA

O.M.Zherelova

Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Moscow Region, 142292, Russia

Ca²⁺ current recordings have been made on Nitella syncarpa cells using the intracellular perfusion and the voltage-clamp technique. TPA (12-O-tetradecanoylphorbol 13-acetate), a substance capable of activating protein kinase C from plasmalemma of Nitella cells, modulates voltage-dependent Ca²⁺ channels. Polymixin B, an inhibitor of protein kinase C, blocks the Nitella plasmalemma Ca²⁺ channels and the rate of channel blocking depends on the concentration and exposure time of the substance.

The blocking of Ca²⁺-current by polymixin B inhibition of protein kinase C supports our assumption that protein kinase C participates in regulation of the activity of Nitella cell plasmalemma Ca²⁺ channels.

Mo-113 DOES ADENOSINE ACTIVATE ATP-SENSITIVE POTASSIUM CURRENTS IN NEONATAL AND ADULT VENTRICULAR CARDIOMYOCYTES?

A.P. Babenko, S.T. Kazantseva and V.O. Samoilov

Laboratory of Biophysics of Living Cell, Military Medical Academy, Saint Petersburg, 194175, Russia

It is hypothesized that cardiomyocyte ATP-sensitive potassium (KATP) channels play an important role in adenosine (AD) effects on heart, since the possibility of coupling A1 receptor with KATP channel via G-protein was shown in experiments on sarcolemma fragments excised from neonatal rat ventricular cardiomyocytes. In the present study we have examined AD-activatability of whole-cell KATP currents in neonatal and adult rat ventricular myocytes in the conditions of intact components of transmembrane signalling. Using amphotericin-perforated patch-clamp technique integral and single channel currents were recorded in whole-cell and perforated vesicle configurations. Magnitude of outward potassium whole-cell current induced by 100 μ M AD was estimated after preliminary addition of 10 μ M acetylcholine (to maximally activate AD-activatable muscarinic potassium channels, which could be present in sarcolemma). KATP channel currents were identified using 5 μ M glibenclamide. Total KATP conductance of sarcolemma was determined when myocytes were subjected to metabolic inhibition with 0.7 μ M FCCP and 7 μ M rotenone in glucose-free extracellular solution to maximally activate KATP channels. Sarcolemma capacitance was estimated and the channel density in membrane was calculated. Despite KATP channels were presented in all cells (20 ± 11 and 35 ± 14 channel/pF in neonatal and adult myocytes respectively), slight AD-activated KATP component was recorded only in two neonatal myocytes possessing small acetylcholine-activated potassium currents (21 neonatal and 18 adult cells were investigated). The AD-activatable KATP channels were in the range of 5%. Occasional instances of KATP channels activation by AD were observed in perforated vesicle recordings. The results suggest that AD-receptor-operated KATP channels in ventricular cardiomyocytes do not play a fundamental role in AD effects on heart cells.

Mo-115 IONOPHORE ANTAGONIZES REVERSAL OF MULTIDRUG RESISTANCE

Katalin Goda, László Balkay*, Zoltán Krasznai, James L. Weaver*, Teréz Márián*, Rezső Gáspár, Lajos Trón*, Adorján Aszalós* and Gábor Szabó jr.

Department of Biophysics and *PET Center, University Medical School of Debrecen, 4012 Debrecen, Hungary; *DRT, FDA, Washington, USA.

The possible roles of pH_i and transmembrane potential in the P170 protein-related multidrug resistance (MDR) have been investigated, studying the effects of various ionophores on pump activity. We attempted to address this question in a systematic manner, examining the cellular accumulation of three fluorescent MDR substrates, daunomycin, rhodamine 123 and Hoechst 33342 (Dm, R123 and H342), with differently pH-dependent physicochemical characteristics and intracellular localization, in several multidrug resistant and sensitive cell lines. Parallel changes of intracellular pH (pH_i), transmembrane potential (Ψ) and drug uptake during ionophore treatments were measured by flow-cytometric and patch-clamp techniques. Surprisingly, reversal of the MDR phenotype by the potassium ionophore valinomycin (Val) was overcome by carbonylcyanide m-chlorophenylhydrazone (CCCP), a mobile proton carrier, in MDR⁺ cells, in the case of R123 and Dm. This effect was not observed on MDR⁻ cells and it was not due to changes of the overall pH_i and/or Ψ , while it was exclusively observed at $\Psi=0$, neutral pH_i and CCCP+Val-dissipated intracellular ion gradients. It is concluded that equilibration of intracellular pH_i and Ψ differences markedly facilitate intracellular drug redistribution, thereby increasing the accessibility of drugs for P170, which presumably extrudes the uncharged membrane-dissolved form of drugs.

Mo-114 EFFECT OF ATP AND THAPSIGARGIN ON THE RELEASE OF STORED Ca²⁺ AND CALCIUM ENTRY IN GLIOMA C6 CELLS.

P.Sabata, M. Czarny, A. Rojek and J. Barańska

Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland.

The effect of ATP and thapsigargin on intracellular Ca²⁺ was investigated using digital video imaging of Fura-2AM loaded into single glioma C6 cells.

We found that in the absence of external calcium, both ATP and thapsigargin induced a rapid calcium release from internal stores, while upon the readdition of external Ca²⁺ an increased rate of calcium influx could be observed in both cases.

In the absence of extracellular calcium the addition of ATP after a transient increase in Ca²⁺, caused by thapsigargin did not produce a further Ca²⁺ response.

These data suggest that in glioma C6 cells ATP and thapsigargin release calcium from the same intracellular pool.

Mo-116 THE ROLE OF Ca²⁺/H⁺ EXCHANGER IN THE CELL.

S.N.KUCHERENKO

Biochemistry of Muscle Department, Institute of Biochemistry, Kiev, Ukraine

Δ pH formation is registered when studying Ca²⁺ passive transport through cardiomyocytes' and lymphocytes' plasma membrane (PM) or through vesicles' membrane made from PM of the sources mentioned above. pH meanings was strongly depended from pH_i. pH changing lead to pH_i somward changing and affect on Ca²⁺ concentration in cytoplasm of intact cells. The presence of Ca²⁺-channels antagonists does not affect this phenomenon. There were linear dependence between the changing of all three values in physiological pH range. Ca²⁺/H⁺ exchanger is proposed to exist in PM. It is also very interesting that cytoplasmic Ca²⁺ and H⁺ activities are some equal in physiological range. Additionally, H⁺-buffering as well as Ca²⁺-buffering systems present in the cell and have their maximal capacity about 7.2 in intact cells. The spectrofluorimetric studying of internal lymphocytes' H⁺-buffering capacity with titration technique using weak base, acid or other buffer addition gave maximal value of 9±1.09 mM depending on what substance to be added. Exchanger is operated only by Ca²⁺ or H⁺ gradients through PM and exhibit their kinetic constants. Short-term cells treatment by trypsin in the conditions to preserve and block Ca²⁺-transporting systems strongly impact on their meanings. We determined K_m and V_{max} both for Ca²⁺ and for H⁺ on both sides of PM. They differed for cardiomyocytes and lymphocytes. Obtained constants said that Ca²⁺/H⁺ exchange process goes to Ca²⁺ influx in change to H⁺ efflux in intact cells. Organism's some states as well as some intracellular events, ordinary or pathologic, may proceed with cytoplasmic pH changing that itself lead to Ca²⁺ concentration changing and to Ca²⁺-depended processes and enzymes' activity changing. We showed, for example, that glucose starvation in incubation medium resulted in pH falling and intracellular Ca²⁺ raising

**CALCIUM INVOLVEMENT IN ELICITATION OF
Mo - 117 PHYTOALEXIN SYNTHESIS IN *Allium cepa*
CELL CULTURE**

J.V.Dyachok, A.P.Dmitriev

Laboratory of Plant Disease Resistance, Institute of Cell Biology and Genetic Engineering, 148 Zabolotnogo St., Kiev, Ukraine

Elicitation of plant defence reactions includes the "recognition" between plant and pathogen. Two phytoalexins (PA) have been identified recently in onion, after infection with patogenic fungi. We are studying the mechanisms of signal perception and transduction using PA as markers of induced disease resistance.

Fast growing callus and suspension cell cultures of onion have been obtained. Conditions of PA synthesis in suspension culture has been investigated. PA accumulation in onion suspension culture induced by pretreatment with biotic elicitor derived from *Botrytis cinerea* culture filtrate.

It has been shown that Ca^{2+} is necessary for the induction of PA accumulation in onion suspension culture. Ca^{2+} -binding by EGTA or blockade of plasmalemma Ca^{2+} -channels by verapamil led to strong decrease of PA accumulation. It has been discovered that agonist Ca^{2+} -ionophore A23187 induced PA accumulation in onion suspension culture.

We conclude that the elicitation of PA synthesis in onion suspension culture depends on external Ca^{2+} accessment.

Mo - 119 COUPLING OF Ca^{2+} - Na^+ TRANSPORT IN RBL-2H3 CELLS AND ITS ROLE IN THE IMMUNOLOGICALLY STIMULATED EXOCYTOSIS

E.Rumpel¹, U.Pilatus², A.Mayer¹, I.Pecht²

¹Dept. of Physics, University of Bremen, Bremen, Germany; ²Dept. of Chem. Immunology, Weizmann Institute of Science, Rehovot, Israel;

The rat mucosal mast cell line RBL-2H3 is widely used for studying the stimulus-secretion coupling process. In these cells stimulation with antigen causes a rapid initial increase of cytoplasmic $[\text{Ca}^{2+}]$ due to its release from intracellular stores. However, optimal secretion requires a sustained elevated $[\text{Ca}^{2+}]_{\text{in}}$, which is achieved by influx of $[\text{Ca}^{2+}]_{\text{ex}}$ by a mechanism which is still under investigation. Since increased membrane permeability for Na^+ has also been observed, and secretion is at least partially inhibited in the absence of $[\text{Na}^+]_{\text{ex}}$, the involvement of a Na^+ - Ca^{2+} -antiporter has been considered. In order to characterize the role of Na^+ in the Ca^{2+} -influx, we examined the extent of secretion (measured by the amount of released β -hexosaminidase) at different $[\text{Na}^+]_{\text{ex}}$. At $[\text{Na}^+]_{\text{ex}}=0.4\text{mM}$ the secretion was not affected by $[\text{Ca}^{2+}]_{\text{ex}}$, (while it was almost completely inhibited at $[\text{Na}^+]_{\text{ex}}=136\text{mM}$ and $[\text{Ca}^{2+}]_{\text{ex}}<0.05\text{mM}$). With increasing $[\text{Na}^+]_{\text{ex}}$ the secretion decreased to a minimum at 15mM $[\text{Na}^+]_{\text{ex}}$, followed by a steady increase to the maximal value. Parallel studies monitoring $^{45}\text{Ca}^{2+}$ -fluxes, indicate augmented Ca^{2+} -influx into resting cells upon decrease in $[\text{Na}^+]_{\text{ex}}$. Stimulation did not cause any detectable change of $[\text{Ca}^{2+}]_{\text{in}}$ while $[\text{Na}^+]_{\text{ex}}$ was below 15mM . At $[\text{Na}^+]_{\text{ex}}$ between 15mM and 20mM we observed a slight but significant decrease of $[\text{Ca}^{2+}]_{\text{in}}$ caused by stimulation. Above $[\text{Na}^+]_{\text{ex}}=20\text{mM}$ stimulation triggered Ca^{2+} -influx, resulting to the well known elevated $[\text{Ca}^{2+}]_{\text{in}}$. Our results clearly indicate that the Na^+ dependence of the secretion is caused by its influence on $[\text{Ca}^{2+}]_{\text{in}}$ by Ca^{2+} - Na^+ -flux coupling via the Na^+ - Ca^{2+} -antiporter.

GROWTH AND CELL DENSITY DEPENDENT EXPRESSION OF STATHMIN IN C2 MYOBLASTS IN CULTURE.

A.Balogh and A.Sobel
INSERM U153-CNRS ERS64, Paris, France

Stathmin (also designated p19, oncoprotein 18 or prosolin) is a 19 kDa cytoplasmic phosphoprotein, presumably a general intracellular relay integrating diverse signal transduction pathways (JIBS 16, 301). It is the generic member of a conserved protein family (see abstract by Ozon et al.).

The expression of stathmin decreases during myogenic differentiation of C2 cells. We examined by immunoblotting the level of stathmin during culture of several C2 subclones. When cells were plated at a density of 1500 cells/cm^2 , stathmin was not detectable 24 hours after plating, started to increase at 48 hours to reach its maximum expression after 4-5 days of culture, when the cells became confluent. The expression of stathmin mRNA was also investigated in parallel with the protein. When cells were plated on a tilted culture dish for 30 minutes to increase their initial density on one half of the dish, and then cultured horizontally for 4 days, high levels of stathmin were present in the dense half as compared to the low density side of the dish. The increase of stathmin expression is thus not due to an autocrine factor secreted by the cells, but more likely to direct interactions between cells as their density increases. This is of particular interest in myogenic cells, whose differentiation towards myotubes begins with the close alignment of cells prior to their fusion.

We are currently investigating whether cell contacts are sufficient to trigger high stathmin expression and whether this phenomenon is characteristic of myogenic cells or more general in nature.

Mo - 120 ATP INDUCES Ca^{2+} INCREASE, ACTIVATION OF $\text{K}^+(\text{Ca})$ CHANNELS AND PARTIAL UNCOUPLING BETWEEN PAIRS OF ELECTRICALLY COUPLED HEPATOMA CELLS.

A.Lazrak, A.Peres*, S.Giovannardi* & C.Peracchia. Department of Physiology, University of Rochester, Rochester, NY, and *Dipartimento di Fisiologia e Biochimica Generali, Universita' di Milano, Milano, Italy.

We have tested the effects of the activation of purinergic receptors linked to phosphoinositide turnover on the gap junction conductance between two Novikoff hepatoma cells. Membrane currents, junctional conductance and $[\text{Ca}^{2+}]_{\text{j}}$ were monitored during stimulation with ATP. Treatments with 10 to $100 \mu\text{M}$ ATP gave rise to a Ca^{2+} transient with fast upstroke and slower recovery. This result was observed both with normal $[\text{Ca}^{2+}]_0$ (2 mM) and in zero external Ca^{2+} , indicating that the Ca^{2+} increase results from internal release. Simultaneously, we observed an outward current (at normal $[\text{K}^+]_0$; $V_h=-20 \text{ mV}$) with a reversal potential of -80 mV ; this current became inward at $140 \text{ mM} [\text{K}^+]_0$ ($V_h=-60 \text{ mV}$) with a reversal potential of -8 mV . ATP then induces a Ca^{2+} rise with consequent opening of K^+ channels. This current may be used to electrically monitor Ca^{2+} changes in cell pairs studied by double whole-cell clamp in order to measure the junctional conductance (G_j). G_j decreased by ~30% upon stimulation with $100 \mu\text{M}$ ATP, following the same time course of the outward K^+ current. Current peaks coincided with G_j minima, indicating that the two phenomena are caused by the same factor, namely, an increase in $[\text{Ca}^{2+}]_{\text{j}}$. This suggests that gap junctions participate in cellular functions modulated by second messengers. Supported by NIH GM20113 to C.P. and by CNR CT92.00730.CT04.115.11537 to A.P. and C.P.

Mo-121

ω -CONOTOXIN AND Cd²⁺ STIMULATE THE RECRUITMENT OF AN INTRACELLULAR POOL OF VOLTAGE-OPERATED CALCIUM CHANNELS TO THE PLASMA MEMBRANE OF NEURONAL CELLS

Maria Passafaro^{a*}, Francesco Clementi^a Emilio

Carbone[#] and Emanuele Sher^c ^aCNR Center of Cytopharmacology, Dept. of Medical Pharmacology, University of Milan, Milan; ^bDept. of Biology, Faculty of Science, University of Rome "Tor Vergata", Rome and ^cDept. of Human Anatomy and Physiology, University of Turin, Turin Italy.

ω -Conotoxin GVIA (wctx) specifically blocks a subtype of voltage-operated calcium channel (VOCC). ¹²⁵I-wctx binding to living neuroblastoma cells at both 4°C and 20°C revealed a low number of surface binding sites (5-10 fmoles/mg of protein) which did not significantly increase with time. On the contrary, ¹²⁵I-wctx binding at 37°C continuously increased, reaching a plateau after 6-8 hours which was up to six times higher than that observed at lower temperatures. The same effect was induced by short (<30 min) pulses with unlabelled wctx followed by a chase of 1-5 hours at 37°C in control medium. Treating the cells with Cd²⁺, an inorganic VOCC blocker, also induced the up-regulation of surface ¹²⁵I-wctx binding sites. Fura2 and patch clamp experiments showed that the recruited binding sites corresponded to functionally active VOCCs. Experiments on permeabilized cells revealed the presence of a large intracellular pool of ¹²⁵I-wctx binding sites, which decreased in size as surface recruitment progressed. VOCC recruitment to the plasma membrane was temperature and energy dependent, but independent of protein synthesis. Brefeldin A (BFA) and nocodazole both prevented this transport in a dose dependent manner. These data suggest a subcellular compartmentalisation of VOCCs in neuronal cells, and that specific stimuli might induce VOCC translocation to the plasma membrane and, in this way, modulate pre-synaptic events.

COUPLING OF IONIC FLUXES IN NUCLEAR MEMBRANE PORES

Mo-123 L.V. Schagina, A.E. Grinfeldt, A.A. Lev

Institute of Cytology of the Russian Academy of Sciences, 4 Tichoretsky avenue, 194064 Saint-Petersburg, Russia

Recent studies on polyethylene terephthalate nuclear filters showed many characteristics peculiar to ionic channels of cell membranes: discrete conductance fluctuations, high ionic selectivity, inhibition of the conductance by protons and divalent cations [1,2]. Our isotope studies on the nuclear membranes with 10 nm of diameter pores and $1.2 \cdot 10^6 \text{ cm}^{-2}$ pore density revealed high cation selectivity (i_+^+ up to 0.9) and Ussing's flux ratio exponent n equal to 1.1-1.2 for Rb⁺ and Na⁺ ions and to 0.6 for Cl⁻ ions (0.01-0.1 M solutions of RbCl or NaCl, pH 6.0). Drop in pH to 1.9 resulted in the conversion of cation selectivity of the membranes to anion one ($i_+^+ = 0.25$). In that case the n values increased for Cl⁻ ions up to 1.1 and decreased for Na⁺ ions to 0.8-0.9.

Our experiments showed ion flux coupling being negative ($n < 1$) that is commonly considered as an evidence of mobile carrier mediated exchange diffusion may be a characteristic of ion transport through a narrow pore.

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Mo-122

DISTRIBUTION OF CYTOSOLIC FREE CALCIUM [Ca²⁺] IN THE UNICELLULAR GREEN ALGA *MICRASTERIAS*

A. Holzinger^a, P. Hepler^b and U. Meindl^a

^aUniversity of Salzburg, Institute for Plant Physiology, 5020 SALZBURG, Austria; ^bUniversity of Massachusetts, Biology Department, AMHERST, MA 01003, USA

Development of the unicellular desmid *Micrasterias* is characterized by a multipolar tip growth and the formation of a complicated symmetrical cell pattern. It has been found that there are inward ionic currents in the areas of the growing lobes which are carried at least in part by calcium. The zones of accumulated calcium are limited to the actual growth areas of the cell and change during the course of cell development.

Since it was speculated that this local influx of calcium would cause gradients in cytosolic free calcium [Ca²⁺] we investigated the calcium distribution in growing *Micrasterias* cells. By microinjecting dibromo-BAPTA, which is supposed to disturb a possible [Ca²⁺] gradient, into growing half cells, we found that there was no effect on pattern formation. Therefore measurements of [Ca²⁺] concentration were carried out by injecting fura-2-dextran and subsequent ratio imaging. With this method we observed that [Ca²⁺] is evenly distributed or only slightly elevated at the very tips of the cell. This means that there is no or no detectable [Ca²⁺] gradient during multipolar growth of *Micrasterias*. Also, treatment with caffeine leads to malformations of the pattern only when applied in high concentrations (50mM).

Ca_i CALMODULIN AND PROTEIN KINASE C INVOLVEMENT IN THE REGULATION OF SODIUM CONDUCTANCE IN *Necturus* ANTRUM

Mo-124 P. Bakos, E. Frömler¹

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic,
¹Zentrum der Physiologie, Klinikum der J.W. Goethe Universität, Frankfurt/M., Federal Republic of Germany.

Antrum mucosa of *Necturus maculosus* is known to transport sodium across amiloride-sensitive sodium channels, but the physiological significance and control of this Na⁺ conductance is not yet well understood. Thus far only feeding has been recognized as a stimulant.

We noticed that antrum mucosae, freshly mounted in Ussing chambers did not exhibit any amiloride-sensitive transepithelial voltage (V_t) or short-circuit current (I_{sc}). Within 1 to 2 hours, however, amiloride (10^{-6} M) sensitivity developed ($\Delta V_t = -10.8 \pm 3.7 \text{ mV}$, $\Delta I_{sc} = 6.0 \pm 3.2 \mu\text{A/cm}^2$ - fasted animals; $\Delta V_t = -26.5 \pm 9.5 \text{ mV}$, $\Delta I_{sc} = 20.8 \pm 4.53 \mu\text{A/cm}^2$ - fed animals), suggesting activation of apical cell membrane Na⁺ channels. Trying to understand this phenomenon we have studied the influence of ion substitutions, hormones, transmitters and drugs that affect intracellular signal transduction. Basolateral ouabain (10^{-6} M), Na⁺ substitution, ionomycin (10^{-6} M), phorbol 12-myristate 13-acetate (10^{-6} M) and trifluoperazine (10^{-6} M) prevented the development of amiloride-sensitive ΔV_t or inhibited I_{sc} , while stimulating the cAMP or cGMP pathway had no effect. We conclude that apical Na⁺ conductance of *Necturus* antrum mucosa is regulated through cell calcium both via protein kinase C and via calmodulin dependent protein kinase. However, which signal triggers the presumed change in cell calcium after tissue isolation is not yet known.

THE SIGNIFICANCE OF CALCIUM OVERLOAD IN
NEURONAL CELL DAMAGE

Mo-125

P. Jalč

Institute of Neurobiology SAV, Košice, Slovakia

It has been assumed, that one of the main causes of destruction of cells damaged by ischaemia, of the transition from reversible to irreversible changes, are calcium ions. Massive calcium overload is noted in neuron irreversibly injured by ischemia and then reperfused with arterial blood. Calcium overload is also a feature of catecholamine cardiotoxicity, the calcium paradox phenomenon, and prolonged high flow substrate-free²⁺ anoxia in vitro. In each of these instances, Ca²⁺ enters the cell, where it is massively accumulated by mitochondria. In addition, in the affected cells, cell membranes are ruptured, and cell contents are lost in the extracellular space. Presumably, many of these potentially lethal changes occur because of unrestricted entry of calcium into the cell. These observations have led to the speculation that calcium overload per se may be a lethal event in ischemic injury. However, it seems likely that the calcium overload occurs as a result of a change in membrane function or structure. Thus, the membrane changes might better be considered the primary or lethal event. The problem in distinguishing the primary event is the fact that it is difficult to disassociate Ca entry from membrane damage. To study this problem we have measured calcium sequestration in hippocampus 2½ h after 30 min ischemized rat

IMMUNOCYTOCHEMICAL LOCALIZATION OF A VACUOLAR ATPase IN THE MALPIGHIAN TUBULES OF TWO INSECT SPECIES (*Formica polyctena* and *Locusta migratoria*)

Mo-127

M. J. Lezaun, M. Garayoa, A. C. Villaro, L. Montuenga and P. Sesma.

Departamento de Histología y Anatomía Patológica, Facultades de Medicina y Ciencias, Universidad de Navarra, 31080 Pamplona, Spain.

Vacuolar ATPases (V-ATPases) are proton pumps comprised of multiple subunits. Though V-ATPases were first mainly associated to endomembranes of acidic organelles, evidence is accumulating for the presence of these enzymes in the plasma membrane of specialized types of vertebrate and insect cells. The presence of a V-ATPase in the Malpighian tubules of two insect species: *Formica polyctena* (Hymenoptera) and *Locusta migratoria* (Orthoptera), has been immunocytochemically investigated using antibodies to *Manduca sexta* midgut V-ATPase and bovine kidney V-ATPase.

Specific labelling was observed at the brush border of the Malpighian tubules. In *Formica polyctena* only one cell type is present, and the immunoreactivity extended along the whole length of the tubules; in *Locusta migratoria*, however, immunolabelling was observed at the brush border of principal cells but not on mucocytes.

These findings are in agreement with the current view that a V-ATPase is situated at the apical membrane of Malpighian tubule cells, energizing the active transport of K⁺ and/or Na⁺ into the lumen.

This study was supported by a grant from the EC (SCI-CT90-0480), and from the Ministerio de Educación y Ciencia (DGICYT, CE 91-0002).

ANION EXCHANGER IMMUNOREACTIVITY IN HUMAN SALIVARY GLANDS

Maria Vázquez, J. Jaime Vázquez, L. Montuenga, Eduardo Martínez* and Jesús Prieto*.

Mo-126 Department of Histology and Pathology; * Department of Internal Medicine, School of Medicine, University of Navarra. Spain.

Salivary gland ducts play a relevant role in saliva secretion. The primary secretion elaborated by the end-pieces is modified in the ducts by transport processes. Na⁺-independent chloride-bicarbonate anion exchangers (AE) may be involved in the generation of ion fluxes into the salivary secretion. Immunohistochemical study was carried out in parotid, submandibular and labial salivary glands from normal subjects. The specimens were fixed in 10 % neutral formaline. The two primary antibodies to detect AE immunoreactivities were prepared in our lab.

AE2 immunoreactivity was restricted to the epithelium of the ducts, with no staining at the acini. A strong positivity was seen in the basolateral portion of the striated ducts, where the interdigitating cytoplasmic processes of adjacent cells come together, while a weak positivity was observed in the interlobular ducts throughout the plasma membrane, including the apical region. AE1 immunoreactivity was only located at the erythrocyte membranes.

In conclusion AE2 immunoreactivity is found in the ducts of salivary glands, particularly in the striated ducts. Previous physiological findings suggests that Na⁺-independent Cl⁻-HCO₃⁻ exchange through the basolateral plasma membrane plays a relevant role in the control of intracellular pH and in the secretion of HCO₃⁻. Our results support the hypothesis that AE2 protein is the carrier for the salivary Na⁺-independent Cl⁻-HCO₃⁻ exchange, and that this exchange takes place in the duct cells rather than in the acinar cells.

This study is supported by grants from the "Dirección General de Investigación Científica y Técnica" (DGICYT), project nº PB92-1133, and the "Foundation Ramón Areces", through the "Center of Biomedical Research". (M. Vázquez).

IMMUNOCYTOCHEMICAL LOCALIZATION OF A VACUOLAR-TYPE ATPase IN THE HINDGUT OF TWO INSECT SPECIES: *Formica polyctena* and *Locusta migratoria*.

M. Garayoa, M. J. Lezaun, A.C. Villaro, L. Montuenga and P. Sesma.

Departamento de Histología y Anatomía Patológica, Facultades de Medicina y Ciencias, Universidad de Navarra, 31080 Pamplona Spain.

Over last years, evidence is accumulating for the presence of a vacuolar-type H⁺ pump in several insect epithelia in which ions are transported against their electrochemical gradient.

The presence of a vacuolar ATPase (V-ATPase) in the hindgut of two insect species: *Formica polyctena* (Hymenoptera) and *Locusta migratoria* (Orthoptera) has been immunocytochemically investigated using antibodies against *Manduca sexta* midgut V-ATPase and bovine kidney V-ATPase.

Immunoreactivity was found along the ileum and rectum in *Locusta migratoria*; labelling was observed along the ileum in *Formica polyctena*. In both ileum and rectum, the enzyme localization was restricted to the apical region of the epithelial cells, underneath the cuticle.

Our results support the possibility that a V-ATPase would energize secondary active transport of anions that occur in the hindgut epithelia.

This study was supported by a grant from the EC (SCI-CT90-0480), and from the Ministerio de Educación y Ciencia (DGICYT, CE 91-0002).

Mo - 129 Demonstration of calcium transient in *Amoeba proteus* caused by application of K and Na ions in different orders

Wanda Kłopocka and Paweł Pomorski

Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, POLAND.

The changes of intracellular calcium level were measured by a magical image processing system (Applied Imaging Co.). Fura 2, a general indicator of free calcium concentration was used to demonstrate and measure calcium changes in *Amoeba proteus* cells caused by application of two succeeding pinocytic inducers: 125 Mm NaCl and 125 Mm KCl applied in both possible orders. The second inducer was always supplied 20s after the first one. When Na ions were the first inducer, sharp increase in cytoplasmic calcium level was observed a few seconds after application of NaCl as well as KCl as second inducer. When KCl was first applied, the rise of Ca²⁺ concentration was induced, but there was no stimulation after following application of NaCl.

According to Jøssefson et al. (1975) and our earlier results (Kłopocka and Grębecka, 1985), potassium is stronger pinocytic inducer than sodium. It seems that K ions, when applied as a second inducer are able to increase again the level of cytoplasmic calcium by stimulating the Ca²⁺ influx or the depletion of intracellular calcium stores or both.

Kłopocka W. and Grębecka L. (1985) *Protoplasma* Vol. 126, pp. 207-214

Jøssefson J. O., Holmer N. G. and Hansson S. E. (1975) *Acta Physiol. Scand.* Vol. 94, pp. 278-296

NICORANDIL EFFECTS AGAINST EARLY MYOCARDIAL REPERFUSION DAMAGE .

Mo - 131 S.Musat and L.M.Popescu

Department of Cell Biology and Histology,
"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

The cardioprotective effects of Nicorandil, an ATP-operated potassium channel ($I_{K\text{-ATP}}$) opener with nitric oxide donor ability, were evaluated in the isolated perfused rat heart model.

After 30 minutes of normothermic global ischemia, 1 μM Nicorandil at the beginning of reperfusion resulted in a significant improvement of recovery, assessed by intracellular enzyme release (approx. 50 % reduction of LDH and CK activity in effluent, when compared to control values), heart rate and ventricular arrhythmias.

Under Nicorandil, cardiac cycle parameters (ventricular systole and rapid filling intervals) at the end of reperfusion were restored close to the preischemic values (preischemia: 57.2±4.2 ms and 30.1±2 ms, control: 45.1±3.2 ms and 41.6±3.7 ms, Nicorandil: 65±11.7 ms and 37.9±2.8 ms, respectively).

These cardioprotective effects were prevented when 4 μM Glibenclamide (a specific $I_{K\text{-ATP}}$ blocker) was added simultaneously with Nicorandil.

The prolongation of ventricular systole interval, under Nicorandil perfusion, further suggests the involvement of $I_{K\text{-ATP}}$, but nitric oxide dependent mechanism can not be excluded, since Aprikalim (another $I_{K\text{-ATP}}$ opener) did not achieve any protection when given only in reperfusion.

EFFECT OF DEXAMETHASONE ON SODIUM CHANNEL BLOCK IN A6 CELLS

Mo - 130 M. Granitzer, I. Mountian and W. Van Driessche

Lab. of Physiology, KULeuven, Gasthuisberg, B-3000 Leuven, Belgium.

Tight renal Na⁺ reabsorbing epithelia contain Na⁺-specific channels within their apical membranes. These channels, which are specifically inhibited by the diuretic amiloride, mediate Na⁺ entry from the apical side into the cell. Their gating properties are regulated by hormones, Na⁺, pH and G-proteins. Using noise analysis, we investigated the influence of dexamethasone (DEX), -a synthetic analogue of cortisol and promotor of cell differentiation-, on Na⁺ channel block, single channel current, densities and open probability of the Na⁺ channels in A6 cells. Short exposure (3 hr) to 10⁻⁷ M DEX increased the short-circuit current by 81% without a change in the ON and OFF rates of the amiloride-Na⁺ channel interaction. A 24 hr incubation with DEX induced a further rise in short-circuit current with a significant increase in the ON rate of the amiloride-binding. The OFF rate remained stable. Since 24 hr DEX depolarizes the apical membrane, voltage dependency of the ON and OFF blocking rates of amiloride were analysed in the absence of DEX. Transepithelial voltage clamps revealed increased ON and decreased OFF rates with hyperpolarization of the apical membrane. Hence, we expected in fact a decrease in the ON rate after DEX. The data, however, revealed an increase in ON rate of amiloride after 24 hr DEX. ON and OFF rates of the binding of CDPC, a neutral analogue of amiloride, was not affected by 24 hr DEX. Further calculations indicated that the augmentation of the macroscopic current after chronic DEX might be explained by the increase in Na⁺ channel density and to a lesser extent in single channel current. Open probability remained stable.

ATP-SENSITIVE POTASSIUM CHANNEL OPENERS ENHANCE MYOCARDIUM SURVIVAL TO ISCHEMIA/REPERFUSION

Mo - 132 INJURY

TRIFAN O.C., MUSAT S., LEABU M., POPESCU A., CIALACU V., TZIGARET C.M., POPESCU M. and POPESCU L.M.

Department of Cell Biology and Histology,
"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

Isolated Langendorff-perfused rat hearts were subjected to 30 min of ischemia and 30 min of reperfusion. The treatment, 10 min before onset of ischemia, with the ATP-sensitive K⁺ (KATP)-channel opener Aprikalim (RP 52891) or Nicorandil (0.2 - 1 μM): 1. dose-dependently reduced the release of intracellular enzymes (peroxidase, aspartate aminotransferase, lactate dehydrogenase, creatine kinase and α -hydroxybutyrate dehydrogenase) proteins and low-molecular-weight compounds (ATP and lactate) during reperfusion; 2. maintained the myocardial content of phosphatidylcholine and phosphatidylethanolamine and reduced the accumulation of free fatty acids in myocardium; 3. restored the coronary flow and left ventricular contractility ($LV\Delta p/\Delta t$) measured after 30 min of reperfusion; 4. preserved the normal ultrastructure (based on the relative volume of mitochondria and myofilaments) of ventricular fibers. The effect of Aprikalim on enzyme release was significantly diminished by 4 μM Glibenclamide, a KATP-channel blocker. These results show that myocardial survival during ischemia/reperfusion is conditioned through KATP channel dependent mechanism.

**Ca²⁺ IONS MEDIATE THE PHOTORESPONSE OF
Blepharisma CELLS**

Mo - 133 S. Fabczak^a, H. Fabczak^a and P.-S. Song^b

^aDepartment of Cell Biology, Institute of Experimental Biology, Warsaw, Poland;

^bDepartment of Chemistry and Institute for Cellular and Molecular Photobiology, University of Nebraska, Lincoln, NE 68588, USA

Blepharisma japonicum, ciliate protozoan cells, changes their swimming direction (photophobic response) when exposed to the light. Externally applied Ca²⁺ ionophore A23187 markedly enhances cell photosensitivity while Ca-channel blockers, pimozone and diltiazem manifest opposite effect. The cells of *Blepharisma* exhibit resting membrane potential of about -54mV generated mainly by the concentration of the external K⁺. An increase of illumination intensity induces membrane depolarization consisting of transient phase (receptor potential) followed by an action potential typical for other protozoan cells and maintained late phase of depolarization (afterdepolarization). The action spectrum of the receptor potentials in *Blepharisma* matches the adsorption spectrum of the cell pigment, blepharismin. The receptor potentials showed slight dependence on the external concentration of Ca²⁺ ions while the action potential is a function of external Ca²⁺ concentration. The results suggest (a) that blepharismin is a true cell photoreceptor and (b) Ca²⁺ controls the photoreceptor and action potentials in *Blepharisma*.

GROWTH FACTORS INDUCE K⁺ MEMBRANE CURRENTS WITH DIFFERENT PROPERTIES IN CHICKEN EMBRYO FIBROBLASTS

Mo - 134 H. Repp^a, A. Matzek^a, H. Draheim^a, N. Malettke^a, K. Maric and F. Dreyer

Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig-Universität Giessen, Frankfurter Strasse 107, D-35392 Giessen, Germany

Recently, we have shown that the Rous sarcoma virus (RSV)-src-oncogene product, pp60^{V-src}, profoundly alters the K⁺ membrane current properties of chicken embryo fibroblasts (CEFs; Repp et al., PNAS 90: 3403-3407, 1993). Since pp60^{V-src}, which is a non-receptor tyrosine kinase, requires the G-protein p21^{ras} to exert its transforming activity, our aim was to test whether activation of receptor tyrosine kinases (RTKs), known to activate p21^{ras}, also leads to K⁺ current alterations in CEFs. In addition, we tested lysophosphatidic acid (LPA), which activates the Ras signalling pathway by a G-protein-coupled receptor.

CEFs were deprived of serum for 12 h, which reduced the mean K⁺ current amplitude to 110 pA compared to 370 pA in control cells. Application of epidermal growth factor (EGF), platelet derived growth factor AB-chain heterodimer (PDGF-AB), insulin, or LPA induced a 2 - 4-fold increase of the K⁺ current amplitude with a maximum after 2 h. However, the properties of the K⁺ currents showed remarkable differences. EGF or LPA induced a delayed activating K⁺ current that inactivated completely during prolonged depolarization and that closely resembles the K⁺ current of normal CEFs cultured with 5% serum. In contrast, stimulation by insulin or PDGF-AB led to a fast activating K⁺ current that lacked inactivation, resembling the K⁺ current properties of RSV-transformed CEFs. Our results identify K⁺ channels as targets of growth factor signalling by RTKs in CEFs. In addition, we have established a further way of stimulation of K⁺ channel activity in these cells, i.e. via activation of a G-protein-coupled receptor. Our data argue for a subtly differentiated regulation of K⁺ channel activity in CEFs, depending on the pattern of mitogens present in the extracellular medium.

Supported by the Deutsche Forschungsgemeinschaft (SFB 249 and Graduiertenkolleg Molekulare Biologie und Pharmakologie)

INDUCTION OF CELL DEATH BY METAL CHELATORS AND AGENT-SPECIFIC CHANGES OF THE NUCLEUS

Mo - 135 G. Vogt^a, H.-D. Reiss^b, R. Böhm^c and H. Segner^d

^aDepartment of Zoology 1 and ^bDepartment of Cell Biology, University of Heidelberg, 69120 Heidelberg, ^cDepartment of Zoology 2, University of Karlsruhe, 76128 Karlsruhe, ^dDepartment of Environmental Chemistry & Ecotoxicology, Centre for Environmental Research, 04301 Leipzig, Germany

The metal chelating agents 1,10-phenanthroline, neocuproine, mimosine and EDTA induced death of cultured carp hepatocytes when added to the culture medium in a concentration of 0.1 M for 24 h. In all treatments, the course of cytopathology differed significantly from apoptosis and necrosis, the two fundamental schemes of cell death. Chelator-related transformation of the hepatocytes started generally with prominent alterations of the chromatin and affected the cytoplasmic organelles only late. The copper and zinc chelator 1,10-phenanthroline induced condensation of the chromatin into a unique figure composed of a compact central sphere and two surrounding electron dense shells. All of these structures comprised DNA as revealed by confocal laser scanning microscopy. DNA-fluorescence was also found in the perinuclear cytoplasm indicating a partial cleavage of the nuclear DNA. Exposition of the hepatocytes to the specific copper chelator neocuproine resulted in almost complete resolution of the chromatin except of a dense nucleolar mass and some patches of loosely compacted chromatin. Chromatin depleted nuclei displayed a close-meshed filamentous network which extended throughout the entire organelle. The chemical identity of these DNA-free filaments is still unknown. The non-protein amino acid mimosine which chelates primarily ions like Al³⁺, Fe³⁺ and Cu²⁺ induced first condensation of the chromatin into a homogenously distributed network of fibrils and then gradual decondensation. Chromatin condensation was paralleled by the disappearance of histone-fluorescence from the nucleus, proliferation and duplication of the nuclear envelope and segregation of the nucleolus. EDTA which binds preferably ions such as Ca²⁺ and Mg²⁺ resulted in a similar pattern of chromatin condensation and decondensation but the nucleolus was dispersed very early. It is not yet known whether the described nuclear changes of the hepatocytes are caused directly by metal chelation or indirectly by secondary mechanisms.

ULTRASTRUCTURAL EVALUATION OF TRANSCRIPTIONAL ACTIVITY WITH GOLD-RNase AND SILVER STAINING METHODS

Bartel H., Orkisz S.

Department Histology and Embryology, MMA, Łódź, Poland

The nucleoli are highly dynamic structures of interphase cell nuclei of size, shape and ultrastructure are connected with ribosome biogenesis. It was well established that during regeneration after partial hepatectomy of rat liver, distinct ultrastructural changes of nucleoli related to prerRNA synthesis are observed (Orkisz S., Bartel H., Histochemistry, 87, 57, 1978). Recently, attention has been paid on the relationship between silver staining and transcriptional activity of nucleoli (Hubbel H. R., Stain Technol., 285, 60, 1985).

Our studies were aimed at on ultrastructural evaluation of distribution of argyrophilic deposits and RNase-gold complexes over the nucleoli during rat liver regeneration.

The experiments were performed on 30 male Wistar rats weighing about 250 g, subjected to partial hepatectomy according Higgins and Anderson (Arch. Pathol., 12, 186, 1931). The study material was obtained from the remaining lobe 6-18 hr after hepatectomy. Localization of argyrophilic proteins was visualized by one-step Ploton method (Biol. Cell., 43, 225, 1982). The colloidal gold and RNase-gold complexes were prepared according to techniques described previously (Bendayan M., Puvion E., J Ultrastruct. Res., 274, 83, 1983). Ultrastructural sections were examined with Philips EM 300 electron microscope.

After the staining in the control group as well as after hepatectomy, fibrillar centers (FC) and dense fibrillar component (DFC) of nucleoli were intensively covered with silver grains. Six and nine hrs after hepatectomy silver staining was of a low density and regularly scattered

ULTRASTRUCTURAL ORGANIZATION OF THE MICRONUCLEI IN IRRADIATED PLANT CELLS

Mo-137

A. Vasilenko and P.G. Sidorenko

Institute of Botany, 254601 Kyiv, Ukraine

In this study we tried to extend the electron microscopic analysis to determine the differences in structural peculiarities of the radiation-induced micronuclei formed in plant cells after particle irradiation. It was shown that the organization of some part of the micronuclei did not considerably differ from the former nuclei, as well, in some cases the micronuclei differed substantially, the nuclear matrix of which was characterized by the presence of fully condensed chromatin and the membrane-like inclusions. Such micronuclei are explained to be probably formed by fragments of the chromosomes without a nuclear organizing region and centromere sequences, since they arise from the chromosomal fragments or chromosomes that are not incorporated into daughter nuclei during mitosis. The submicroscopic analysis gives the possibility to reveal both the distinctive chromosomal segment damages as the formation of the micronuclei with the condensed chromatin that is apparently bound up with an yield of accentric fragments of chromosomes, and the segregation-enable micronuclei with a well-defined nucleolus and a diffuse chromatin that are probably formed from the segments with centromere sequences.

EFFECT OF LETHAL HYBRID RESCUE (*Lhr*) GENE ON THE TRANSCRIPTIVE ACTIVITY PATTERN OF THE X CHROMOSOME(S) IN INTERSPECIFIC HYBRIDS OF *Drosophila melanogaster* AND *D. simulans*

Mo-139

R.N. Chatterjee

Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700019, India

Hybrid males from *Drosophila melanogaster* female x *D. simulans* male die as larvae while hybrid females from the reciprocal cross die as embryos. The hybrid lethality can be prevented by the lethal hybrid rescue gene of *D. simulans* (Watanabe, 1979 : Jpn. J. Genet. 52:1-8). In order to know the effect of the 'rescuing' mutation on the functional properties of the salivary gland X chromosome(s) in hybrid, I have undertaken to analyse the functional morphology and transcriptive activity of the polytene X chromosome in various hybrids ($X^s Y^m$: A^mA^s ; $X^m Y^s$: A^mA^s ; $X^m X^s$: A^mA^s ; $X^m X^m$: A^mA^s ; $X^m X^m X^s$: A^mA^s), using ^{3}H -uridine autoradiography. Our results show that the *melanogaster* X chromosome in the hybrid male ($X^m Y^s$: A^mA^s) displayed typical pale staining, enlarged diameter and higher rate of transcription (nearly two times higher than the individual X chromosome of the female), in presence of one dose *Lhr* mutation. However, in males with *simulans* X chromosome ($X^s Y^m$: A^mA^s) and one dose of *Lhr* mutation show nearly twice as much puffy as the X chromosome of normal male. In females (either in partial hybrid $X^m X^m$: A^mA^s or in full hybrid $X^m X^m$: A^mA^s) the normal properties of the female X chromosomes were unaltered in presence of *Lhr* mutation. Our data further reveal that *Lhr* mutation caused marked induction of ^{3}H -uridine incorporation in some developmental puffs of the hybrid.

INTRANUCLEAR LOCALIZATION OF snRNPs IN OOCYTES AND EARLY EMBRYOS OF PIG

Mo-138

V. Kopečný^a, S. Fakan^b, M. Biggiogera^b, J. Laurincík^c and J. Pivko^c

^aInstitute of Molecular Genetics, Academy of Sciences, Praha, Czech Republic; ^bCentre of Electron Microscopy, University of Lausanne, Switzerland; ^cResearch Institute of Animal Production, Nitra, Slovak Republic

Pig germinal vesicle stage oocytes and 1-4 blastomere embryos isolated at slaughter were investigated by immunoelectron microscopy for the distribution of nucleoplasmic (spliceosomal) snRNPs using specific antibodies and protein A-gold labelling. Among the intranuclear structures which showed a preferential RNP staining only some were labelled by the gold particles. These observations suggested that the snRNPs containing the Sm-complex associated with U_1 , U_2 , U_5 and U_4/U_6 snRNPs concentrated in the "compact" nucleoli⁴ (rounded dense intranuclear masses) and in interchromatin-like granules, in turn often forming spherical aggregates. Such unique pathway of (sn)RNPs in the oocyte/embryo system may explain the specific features of both oocyte and early embryo nuclear morphology. The composition of these particles or bodies reflected probably one of the specific functions of the highly differentiated nuclear architecture of mammalian fertilization, namely that involved in conservation of maternal supply of RNAs and RNPs supporting early embryonic development.

CYTOPHOTOMETRIC INVESTIGATION OF NUCLEOLUS ARGENTOPHILIC PROTEINS IN NUCLEI OF DIFFERENT PLOIDY OF MOSS *TORTULA MURALIS* HEDW.

Mo-140

O. Lobachevska

Department of Plant Ecomorphogenesis, Institute of Ecology of the Carpathians of the Academy of Sciences of Ukraine, 290000 Lviv, Stefanika Street, II, Ukraine

Structural and functional state of chromatin of interphase nuclei was investigated by means of method of detection of argentophilic proteins of nucleoli during silver staining of cells in AgNO_3 . The aim of our investigation was to establish character of correlation between quantity of Ag-proteins, connecting with structure of potentially active chromatin and a level of ploidy. For determination of quantity of Ag-protein the cytophotometry of silver staining nucleoli in cells of *Tortula muralis* ($n = 24$, 48, 96) of different level of ploidy was made. To avoid nonspecific stain cytophotometry was carried out in yellow light of length of wave 572 nm. In nuclei of almost all investigated cells one nucleoli was analysed and only in separate cells of caulinema were discovered 2 tiny compact nucleoli. Results of cytophotometry of interphase nuclei of protonema have shown that quantity of argentophilic proteins increases not proportionally increase of level of ploidy of cells and content of nuclear DNA. It is established insignificant decrease of quantity of stained Ag-proteins only in the most competitive 48-chromosome race of *T. muralis* as transcription is inhibited by actinomycin D.

THE RELATIONSHIP BETWEEN MORPHOLOGICAL FEATURES OF NUCLEOLI AND PROLIFERATION ACTIVITY IN CELL CULTURE

Mo - 141 S. Vyguzinnyi, S. Mameeva
Laboratory of Cell Morphology
Institute of Cytology, RAS,
St. Petersburg, Russia

The relationship among number of AgNOR, nucleolar area, number of nucleoli per nuclei, number of nucleoli touching nucleolar membrane, RNA synthesis and proliferating activity of human fibroblasts, human leukaemia HL - 60, U - 937 cells in culture was investigated. Cell proliferation was administrated by serum deprivation under flow cytometry and autoradiography controls.

The strongest correlation ($r = 0.94$) was measured between index of labelling of fibroblasts and the number of nucleoli touching nuclear membrane. Strong correlation was also found among AgNOR count ($r = 0.84$), nucleolar area ($r = 0.78$) and index of labelling.

The average number of silver grains in interphase nuclei decreased slowly during 10 days and increased quickly during 16 hours while quick decrease and quick increase of cell proliferation occurred. The same pattern of nucleolar behaviour was observed in all cell cultures, although the average number of silver grains were related to cell duplication time.

ARTIFICIAL INDUCTION OF MITOTIC-LIKE PRENUCLEOLAR BODIES IN MATURE INTERPHASE NUCLEI

OA Dudnic, OV Zatsepina
Mo - 142

A.N.Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow, Russia

Recently, it has been shown (Dudnic et al., 1993) that action of the low ionic strength solutions on living interphase HeLa cells may result in the complete unravelling of the nucleoli and leads to the spreading of the certain nucleolar proteins (i.e. B23, fibrillarin, Ag-proteins) over the nucleoplasm. The following replacement of the hypotonic medium onto the isotonic one, causes the formation of numerous roundish RNP-containing bodies scattered throughout the nucleoplasm. Due to their fusion with the nucleolar remnants, reconstitution of the nucleoli is taking place. Similarly to the mitotic pre-nucleolar bodies (mPNBs) regularly seen in telophase cells, PNBs artificially induced in interphase cells (iPNBs) also contain B23, fibrillarin, Ag-proteins, but are devoided UBF. Addition of actinomycin D prevents the coalescence of iPNBs with the reforming nuclei. These findings indicate that iPNBs are indistinguishable from mitotic ones and might be applied for biochemical analysis of nucleogenesis.

STRUCTURAL ASPECTS OF THE PROCESSES LEADING TO POSTMITOTIC NUCLEOCYTO-

Mo - 143 PLASMIC COMPARTMENTATION

R. Bleher and R. Martin

Electron Microscopy Section, University of Ulm, D 89069 Ulm, Germany

Applying electron energy loss spectroscopic and stereological methods, as well as cryo-immunocytochemistry, we studied the translocation of organelles and materials during the postmitotic reformation of the cell nucleus in HeLa-cells. The spectroscopic methods allow the selective visualization of phosphorus-rich structures like ribosomes, nucleosomes or nucleolar components in extremely thin sections at high resolution. In prophase the process of cleavage of the nuclear envelope into vesicles also involves RER- and Golgi cisterns in the cytoplasm. When in metaphase the nucleus has disintegrated larger organelles like membrane vesicles, mitochondria or dense inclusions remain segregated in the cellular periphery, while smaller organelles like ribosomes and dissolved materials mix with the chromosomes. This separation is maintained by the spindle microtubules. When in telophase envelope vesicles attach to the chromatin mass a wave of fusion of vesicles involves also non-envelope vesicles in the cytoplasm. Chromosome-associated proteins become included by the new envelope into the reforming nucleus. This is true also for the nucleolus organizing region, but it is not clear whether other nucleolar components become trapped too. Ribosomes in pockets between the chromatin are expelled before closure of the envelope by unidentified material which fills the spaces between the chromosomes. Sealing of the envelope is a late process. It is the aim of this study to visualize events of these dramatic and complex sorting processes.

IMMUNOCYTOCHEMISTRY AND MICROINJECTION OF ANTIBODIES EMPLOYED FOR ANALYZING THE NUCLEOLAR PROTEIN FIBRILLARIN DURING MOUSE EARLY EMBRYOGENESIS

Mo - 144

P. Esponda and J.M. Cuadros Fernández
Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain.

Fibrillarin is a 34 kD nucleolar protein which is directly involved in pre-rRNA processing. Reports on the role of this protein during mammalian early embryogenesis are scarce in comparison to those undertaken on somatic cells. To ascertain the distribution of fibrillarin during mouse early embryogenesis, we employed three antisera from scleroderma patients which contain antibodies that recognize fibrillarin. Immunocytochemistry under light and electron microscopy demonstrated that fibrillarin was initially detected at the boundary of the nucleolar precursor bodies during the late 2-cell stage. This labelling increased gradually until morula stage, in parallel with the raise of rRNA genes transcription occurring in these stages. In order to explore some roles of fibrillarin during early embryogenesis, we microinjected anti-fibrillarin IgGs in the male pronucleus of one-cell stage mouse embryo. After cell culture, only 23.8% of microinjected embryos reached the blastocyst stage, in contrast with the 60.9% of the embryos microinjected with non immune human IgGs. These results indicated that anti-fibrillarin IgGs produce a partial arrest of mouse early embryogenesis, probably by a modification of the resumption of rRNA synthesis.

**A NEW NUCLEAR SMALL STRUCTURE
WHICH HAS A RELATIONSHIP WITH
PERINUCLEAR INTERMEDIATE FILAMENTS**

H. Kamei

Life Science Research Laboratory, Japan
Tobacco Inc., Yokohama, Japan

A monoclonal antibody (AP435 MAb) was obtained which recognize several (5 to 40) small nuclear dots as well as cytoplasmic intermediate filaments in various mammalian cells under immunofluorescence microscopy. Immunoblotting showed that AP435 MAb reacted with 40 KD protein and intermediate filament proteins such as desmin and vimentin. The dots, located on or in the nuclei, did not co-localize with the structures concerning RNA splicing nor centromeres. Many dots located close to perinuclear intermediate filaments. Dots were, however, observed even in the cells lacking intermediate filaments (subclone of SW13 cells). This suggests that the dots have some fundamental functions, though it is not revealed at present. The dots recognized by AP435 MAb may be a new nuclear structure which can interact with perinuclear intermediate filaments.

CYTOPLASMIC LOCALIZATION OF NUCLEAR LAMIN PROTEINS IN XENOPUS OOCYTES

D. Lourim, G. Seubert, and G. Krohne
Division of Electron Microscopy,
Biocenter, University of Würzburg,
97074 Würzburg, Germany

We have previously demonstrated that Xenopus oocytes, eggs, and early embryos contain two lamin isoforms, lamin L_{III} and L_I, and are associated with separate vesicle populations in meiotic egg extracts. Using affinity purified anti-lamin L_I antibodies, we have recently detected a lamin isoform present in Xenopus oocytes which appears similar to the B-type lamin L_I of somatic cells. In contrast to the exclusive nuclear localization of lamin L_{III}, greater than 90% of lamin L_I, and a small percentage of lamin L_{II}, are detected in a low speed centrifugation pellet derived from oocyte cytoplasms. Sucrose gradient analysis in the presence and absence of nonionic detergent confirms the membrane association of these cytoplasmic lamins, and excludes the possibility that these lamins originate from contaminating follicle cell nuclei. Approximately 20-23% of lamin L_I is associated with a heterogenous vesicle population in meiotic egg extracts. Preliminary biochemical and microscopic analysis suggests the cytoplasmic lamins are in the form of short filaments loosely associated with cortical cytoplasm membrane structures which are distinct from annulate lamellae. The utilization of lamin L_I in the formation of embryonic nuclei, and the mechanism for the cytoplasmic retention of these normally nuclear lamin proteins are being investigated.

Mo-146

DYNAMIC RECOVERY OF PHOTOBLEACHED XENOPUS LAMIN A IN THE NUCLEAR LAMINA

M. Schmidt^a, M. Tschödrich-Rötter^b,
R. Peters^b and G. Krohne^a

^aDivision of Electron Microscopy, Biocenter,
University of Würzburg, D-97074 Würzburg;

^bInstitut für medizinische Physik und
Biophysik, Universität Münster, D-48149,
Germany

Xenopus lamin A fluorescent labeled with 5-IAF exhibited assembly properties in vitro and in vivo indistinguishable from the native protein. 5-IAF-lamin A microinjected into the cytoplasm of 3T3 cells was rapidly transported into the nucleus. Incorporation of the labeled lamin A into the nuclear envelope was detectable 30-60 minutes after microinjection whereas the lamin A CaaX-box mutant (C662 → S) at the same time points exclusively formed intranuclear aggregates. We photobleached the nuclear envelope of 3T3 cells 5-12 hrs after microinjection with 5-IAF-lamin. By utilizing two methods, confocal laser scanning microscopy and single photon counting, a significant recovery of fluorescence was recorded within 10 minutes after photobleaching. Models of lamin mobility within the nuclear lamina based on fluorescence recovery will be discussed.

Mo-148

ULTRASTRUCTURE AND NOR ACTIVITY IN THE CAMBIAL TROPHOBlast CELLS OF THE RAT PLACENTA

T.Zybina, E.Zybina
Institute of Cytology of the Russian
Academy of Sciences, St.-Petersburg

A comparative study has been performed of the arrangement of nucleolar components in relations with NOR activity using electron microscopy and light microscope Ag-NOR silver staining in the course of differentiation of low polyploid trophoblast cells. The trophoblast cells of junctional zone and labyrinth of rat placenta at the 12-14 days of gestation has been studied. Each cell type characterized by specific arrangement of nucleolar components. The nucleolonemal nucleoli prevailed suggesting high level of transcriptional activity. Different quantitative parameters of silver staining has been estimated using the image analyse system "Magiscan". The mean number and total area of the Ag-stained granules in the nucleus as well as the mean total nucleolar area estimated for each ploidy level did not change significantly in the course of placenta development suggesting unchanged level of NOR activity at the studied stages of trophoblast cell differentiation. By contrast, the number and total area of Ag-stained granules in the nucleus increased but was not doubled passing to the next ploidy level. Majority of the cells (80-90 %) contained from 1 to 3 nucleoli, the proportion of cells with different number of nucleoli being unchanged at the different ploidy levels. Association of NORs is suggested to be in relation with switching from the polyploid mitotic cell cycle to the endoreduplication leading to the polyteny.

**ADRENAL NUCLEAR MATRIX AFTER ACTH
STIMULATION MORPHOLOGIC AND**

Mo-149 BIOCHEMICAL STUDY

D. Neves, M. M. Magalhães and M. C. Magalhães
Institute of Histology and Embryology, Faculty of

Medicine and Center of Experimental Morphology of the University of Oporto, 4200 PORTO, PORTUGAL

The nuclear matrix is a residual nucleoskeleton containing steroid hormone receptors and also responsible for chromatin organisation, DNA replication and transcription. Adrenal nuclear matrix previously isolated by Neves et al (1), must be a target for ACTH action on steroidogenesis mechanism. In the present study, two experimental groups of male rats (Wistar, Gulbenkian, Oeiras) weighing 170-200g were used. Group I - ten animals were injected with 20 IU/Kg ip ACTH Armour 45 min before sacrifice; Group II - six control animals were injected ip with 0.1 ml serum saline 45 min before death. After sacrifice, by decapitation, the adrenals were removed and decapsulated. The method of Kaufmann et al (2) was used for nuclear matrix isolation which was applied to nuclei purified by the method of Blobel and Potter (3). Nuclei and nuclear matrix fractions were fixed with glutaraldehyde for EM. Proteins and residual DNA were quantified by spectrophotometric methods. Nuclei and nuclear matrix peptides were analysed by SDS-PAGE, and the identification of nuclear lamins A and C, coilin and fibrillarin by Western blotting. For the assessment of serum corticosterone the HPLC technique was used. Nuclei and nuclear matrix of controls (Group II) were well preserved and purified, and nucleolus exhibited a reticular type. Group I animals showed nuclei with less electronodense nucleoplasm and nuclear matrix with a more dispersed fibrilgranular network. No significant peptide differences were observed in either group with the SDS-PAGE technique. In all fractions studied, and in both groups, two proteins of 65 and 80 kd, lamins A and C, respectively, were detected by Western blotting. Fibrillarin (34kd) was detected in both nuclear and nuclear matrix fractions of Group II animals. Coilin (80kd) was detected in less quantity in ACTH treated nuclear matrix (Group I).

(1) Neves D et al (1993) *Biol Cell* 79, 139-145; (2) Kaufmann et al (1981) *Exp Cell Res* 132, 105-123; (3) Blobel G, Potter VR (1966) *Science* 154, 1662-1665;

**AVIAN CHROMOSOMES IN THE LAMPBRUSH FORM:
MORPHO-FUNCTIONAL AND CYTOGENETIC ASPECTS**

Mo-151

E. Gaginskaya, A. Rodionov and I. Solovei

Laboratory of Chromosome Structure & Function, Biological Institute, University of Saint-Petersburg, Russia

Examination of lampbrush chromosomes in a few species of birds have been done with a special attention to site-specific chromosome markers, their chromosomal localization, ultrastructure and cytochemistry. Cytological working maps of all chicken lampbrush macrobivalents, including the asymmetric ZW sex bivalent are constructed. The maps serve as a basis for physical mapping of genes and gene transcription by the *in situ* hybridization technique and for immunocytochemical localization of proteins on avian lampbrush chromosomes. Avian lampbrush chromosomes, also, are considered as an excellent model object for a general exploration into the eukaryotic genome structure and functions.

A series of site-specific landmarks of avian lampbrushes were shown to correlate with GC-enriched chromatin C-bands, the characteristic being related to avian genome and karyotype organization. Specific morpho-functional characteristics of telomeric and centromeric chromosome regions were studied. A novel type of protein structure ("spaghetti marker") associated with chromosome 2 in the chicken lampbrush complement has been discovered and examined. Two families of W chromosome-specific repeated sequences and (TTAGGG)_n telomeric repeat are investigated on chicken lampbrush chromosomes by the fluorescent *in situ* hybridization. Comparative study of sex chromosomes in the lampbrush form was carried out for six avian species.

Mo-150

**THE REVERSIBLE NUCLEO-CYTOPLASMIC
TRANSLOCATION OF THE HIV-1 REV
PROTEIN DEPENDS ON THE ACTIVATION
DOMAIN OF THE PROTEIN**

B. Wolff, G. Cohen, D. Meshcheryakova, J. Hauber and C. Rabeck

Sandoz Forschungsinstitut, Brunner Strasse 59, A-1238 Vienna, Austria

The HIV-1 regulatory protein Rev, which is required for the cytoplasmic expression of unspliced and incompletely spliced viral mRNAs, is located predominantly in the nucleolus. In this study, we show that Rev protein translocation (nucleolus to cytoplasm) is induced in transfected HeLa and COS cells under conditions where rRNA synthesis is inhibited (e.g. with actinomycin D or camptothecin). Upon removal of the inhibitor, Rev re-enters the nucleolus, suggesting that the protein can shuttle between nucleus and cytoplasm.

Dominant-negative mutants with mutations in the activation domain of Rev, which are known to inhibit Rev function in *trans*, are unable to leave the nucleus upon actinomycin D treatment. More importantly, when present in excess, these mutants inhibit the translocation of wild-type Rev. This correlation of inhibitory activities suggests that Rev function depends on its transport to and presence (at least transient) in the cytoplasm. Therefore, nucleo-cytoplasmic export of Rev could potentially serve as a novel target for anti-HIV chemotherapy.

**NUCLEAR IMPORT OF RIBOSOMAL
PROTEINS IN YEAST**

Mo-152

J. van 't Riet, M.F. Feenstra, M.S. Guedes, A.C.J. Timmers and H.A. Raué

Department of Biochemistry and Molecular Biology, Institute for Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Ribosomal proteins (rproteins) are synthesized in the cytoplasm and subsequently transferred to the nucleus for assembly with (pre)rRNA to form the 40S and 60S subunits. Hence the newly formed subunits are exported to the cytoplasm to function in translation. The stoichiometric synthesis of all 80 rproteins is identically regulated at the transcriptional level. Since about 40 ribosomes are formed per second in a yeast cell growing with a doubling time of 90 min the nuclear import of rproteins must be very efficient. Some rproteins however, like L25 and S24, are early assembling proteins (primary binders) and others, like S31, assemble with pre-subunits in a late stage of the assembly process. We wanted to know *i*. whether the rproteins are imported via a specific import mechanism as distinct from that of other nuclear proteins, and *ii*. whether the different classes of rproteins have a different nuclear uptake efficiency. For that we have analyzed the Nuclear Localization Signals (NLSs) of yeast rproteins L25, S24 and S31. It was found that NLSs of rproteins vary as much in amino acid sequence as nuclear proteins generally do and that they represent all the different consensus sequences postulated for NLSs thus far. So no specialized nuclear uptake mechanism seems to exist for rproteins. Neither was the presence and repetitiveness of strong or weak NLSs indicative for a rprotein to be an early or late assembling rprotein, respectively. Since rproteins assemble with (pre-)rRNA in the nucleolus, we have also been looking for nucleolar localization sequences (NoLSs) in rproteins. Though nucleolar localization of fusion proteins, consisting of parts of the rproteins and a reporter protein, depended on the nature of the reporter used no NoLSs have been found thus far, indicating that nucleolar targeting information may reside in the rRNA binding domains of rproteins.

CALMODULIN CAN MODULATE THE PHOSPHORYLATION OF RAT LIVER HNRNP Mo-153 PROTEINS A2 AND C

R. Bosser and O. Bachs.

Dept. of Cell Biology, Faculty of Medicine, University of Barcelona, Barcelona, Spain.

We previously described that calmodulin modulates the casein kinase 2-dependent phosphorylation of three nuclear proteins of 50, 40-42 and 37 kDa which can be extracted from rat liver cell nuclei by nuclease digestion. In order to identify these casein kinase 2 substrates, specific polyclonal antibodies against p37 were raised by immunizing rabbits with the electroeluted protein from SDS gels. Immunoblot and immunocytochemistry experiments using the affinity purified antibodies revealed that p37 is exclusively located in the nucleus but not in the nucleolus. Further experiments using ssDNA overlay and ssDNA-cellulose chromatography showed that p37 tightly binds to ssDNA. All these data suggested that p37 was a hnRNP protein. Two-dimensional immunoblotting with monoclonal antibodies against HeLa hnRNP revealed that p37 protein is the hnRNP A2 protein. The 40-42 kDa substrates have been identified as the hnRNP C protein. The mechanism by which calmodulin inhibits the phosphorylation of these hnRNP proteins by casein kinase 2 is still unclear. However, the binding of these hnRNP proteins to a calmodulin-Sepharose column in a Ca²⁺ dependent manner suggests that calmodulin could specifically block the putative phosphorylatable sites and thus modify the interaction of these proteins with RNA.

A NEW MONOCLONAL ANTIBODY THAT RECOGNIZES HETEROCHROMATIN IN THE NEMATODE PARASCARIS.

G. Giovinazzo^a, M. R. Esteban^a, A de la Hera^b and C. Goday^a

^a Departments of Cell Biology and Development and ^b Immunology. Centro de Investigaciones Biológicas. Velázquez 144, CSIC. 28006 Madrid. Spain.

We have produced a panel of monoclonal antibodies that recognize different nuclear components using total extracts of *Parascaris* embryos as immunogen. We report the cytological and biochemical characterization of a *Parascaris* antigen that associates to heterochromatin and is recognized by mAb 661. By indirect immunofluorescence we show that this antigen specifically associates to the AT-rich satellite heterochromatic regions in *Parascaris* chromosomes. In addition, mAb 661 recognizes heterochromatic regions along the entire cell cycle in different *Parascaris* cell types.

We are currently investigating the presence of similar antigens that are recognized by mAb 661 in distant species as human cells, mouse, *Caenorhabditis elegans*, *Drosophila melanogaster* and plants.

IDENTIFICATION OF PROTEINS THAT BIND TO SATELLITE DNA SEQUENCES IN *Drosophila melanogaster*

Mo-154

Laura Lasseri and Claudio Pisano

Dip. di Genetica e Biologia Molecolare e Centro di Genetica Evoluzionistica; Università "La Sapienza", Roma.

The heterochromatic Y chromosome of *Drosophila melanogaster* carries six genes essential for male fertility (Gatti and Pimpinelli, 1983). Three of them develop in meiotic prophase large jumpbrush-like loops which are arranged into nucleoprotein complexes (Bonacorsi et al., 1988; Bonacorsi et al. 1990; Pisano et al., 1993).

Here, we have worked mainly with proteins that specifically interact with two different cloned sequences of satellite DNA mapping in the loop-forming regions. To this end a series of South-Western blotting experiments have been performed: crude extracts of larval testes, embryos, larval ganglia and salivary glands were fractionated by SDS polyacrylamide gel electrophoresis, proteins were then transferred to nitrocellulose filters and incubated with ³²P terminally labeled AATAT or AAGAG repeats (cloned from the 1.672 and 1.705 *D. melanogaster* satellite DNAs respectively).

Under these conditions both the AATAT and the AAGAG probes bind a 110 kDa somatic protein, which is not detectable in testes. The AATAT probe also recognizes a 62 kDa protein present only in testes and a 58 kDa protein present in all somatic tissues analyzed. The 62 kDa appears to be absent in mature sperm, suggesting that it is not involved in sperm chromatin condensation. Additionally, we found that the binding of both the 62 and 58 kDa proteins to DNA is inhibited by Hoechst 33258, and this is consistent with the idea that they may bind to the minor groove of the double helix. Finally, we found that these proteins are shared also by other *Drosophila* species, such as *D. simulans* and *D. virilis*. Instead, the 62 and 58 kDa proteins appear to be absent in *D. hydei*. These data, together with the evidence that the tested satellite DNA is absent in *D. hydei*, have lead us to the suggestion that some sort of coevolution between AATAT sequence and the 58 and 62 kDa proteins may be occurred.

In addition we have observed several proteins that binds a single strand DNA at more low molecular weight and in particular tree bands at 53, 47 and 35 kDa. We have induced in mouse polyclonal antibodies against these DNA or RNA binding protein. In particular, the antiserum against the 53 kDa shows a strong reaction on the salivary glands and on ks-1 Loop in primary spermatocytes. In bidimensional western this antiserum recognize one single spot with isoelectric point at 6.3. Currently, we are devoting our efforts to the cloning of the genes codifying for these proteins.

Novel mammalian germ line-specific protein: a role in long term inactivation of chromosome domains?

Avril Smith and Ricardo Benavente

Department for Cell and Developmental Biology, Theodor-Boveri-Institute, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

By using the novel monoclonal antibody Mab 4EC raised against proteins of rat spermatogenic cells we were able to identify a germ line nuclear protein of 51 kDa. In Western blot experiments this protein was found to be highly enriched in karyoskeletal fractions of spermatocytes and spermatids. In contrast, somatic cell types tested showed no reaction. Immunocytochemical analysis of rat and mouse spermatogenic cells using Mab 4EC showed that the 51 kDa protein is located in chromatin regions known to be largely transcriptionally inactive. Somatic cells of testes and other tissues are negative.

We speculate that this 51 kDa protein may play a role in regulating transcriptional activity during mammalian gametogenesis. According to our immunocytochemical and cell fractionation data this protein might be involved in mechanisms leading to long term inactivation of certain chromosome domains. The results will be discussed in relation to models previously proposed by other authors dealing with regulation of the functional status of constitutive and facultative heterochromatin.

CLONING OF RCC1/RAN INTERACTING PROTEINS

Mo - 157

L. Mueller and H. Ponstingl

Division for Molecular Biology of Mitosis, German Cancer Research Center, Im Neuenheimer Feld 280, D-69009 Heidelberg, Germany

The nuclear protein RCC1 (Regulator of Chromosome Condensation) is a guanine nucleotide exchange factor (GNEF) of the nuclear ras-related GTPase Ran. RCC1 and Ran are evolutionary conserved molecules and were shown to be involved in the regulation of cell cycle progression and cell cycle control, mRNA metabolism and more recently in nuclear transport in various organisms. In order to identify human proteins involved in the RCC1-regulatory pathway(s) we used the 2-hybrid cloning system in yeast. Human *RCC1* as well as *TC4* (Ran) open reading frames were fused to the GAL4 DNA binding domain and used as "baits" to screen a human cDNA library fused to the GAL4 transactivator domain. Of yeast colonies selected for restored transcriptional activation from the GAL4 upstream activator sequence, four different groups of cDNAs were isolated for the RCC1-bait so far. As expected, the RCC1 substrate Ran(TC4) was shown to interact with RCC1.

EXPRESSION OF CYTOKERATINS FILAMENTS IN THE INNER CELL MASS OF Mo - 159 PARTHENOGENETIC EMBRYOS

Jose A. Uranga and Juan Arechaga. Dept. Cell Biol. Univ. of the Basque Country. 48940 Leioa. Vizcaya. Spain.

The formation of the expanded blastocyst marks the earliest establishment of two distinct and committed cell populations in the mammalian embryo: the trophectoderm and the inner cell mass (ICM), that differ in their properties, developmental potentials and fates. The trophectoderm, the first embryonal epithelium, will differentiate into extraembryonic membranes and the ICM cells will rise to the embryo proper. Mouse parthenogenetic embryos complete preimplantation development and initiate implantation but fail to differentiate normally. Both set of chromosomes are needed because of the action of certain imprinted genes which make maternal and parental genomes complementary. Cytokeratins constitute a family of proteins whose expression is modulated during blastocyst formation being restricted in normal embryos to the trophectoderm but not of the ICM cells. We have used the TROMA-1 monoclonal antibody directed against cytokeratin ENDO A to undertake an indirect immunofluorescence analysis, under confocal laser microscopy, of the presence of assembled protein in normally fertilized and parthenogenetic embryos. Control embryos show positivity located in the trophectoderm and in the primitive endoderm as previously reported. In parthenogenetic embryos however cytokeratin filaments appear clearly both in the trophectoderm and in the ICM cells. This result suggest that also in preimplantation embryos the paternal genome exerts a fundamental action by regulating ENDO A expression.

This work has been supported by grants from DGICYT (Ministry of Education and Science, Spain), Basque Government and Univ. of the Basque Country.

ROLES OF NONRIBOSOMAL NUCLEOLAR PROTEINS IN RIBOSOME BIOGENESIS AND PROTEIN TRANSPORT

Mo - 158

M.O.J. Olson, D. Wang, A. Szebeni, J. Herrera and M. Dundr

Biochemistry Department, University of Mississippi Medical Center, Jackson, MS 39216 U.S.A.

Protein B23 and nucleolin are abundant acidic nucleolar phosphoproteins and putative ribosome assembly factors that shuttle between the nucleus and cytoplasm. Recent work has focused on defining the activities of protein B23. Protein B23 exists as two isoforms, B23.1 and B23.2, which are polypeptides of 292 and 257 amino acids, respectively, differing only at their C-terminal ends. Protein B23.1 is expressed at much higher levels than B23.2 in all cells examined. In cell fractionation experiments protein B23.1 was localized almost exclusively to the nucleolus whereas B23.2 was found in other cellular compartments. The two B23 isoforms bound peptides containing the SV40 T-antigen nuclear localization signal (NLS) with approximately equal affinity. Both isoforms also contained Ca^{++} -stimulated ribonuclease activity. However, only B23.1 bound nucleic acids. A fragment derived from the C-terminal end of B23.1 also had nucleic acid binding activity. This suggests that the nucleolar localization of B23.1 is due to interactions with nucleic acids mediated by its C-terminal end. Protein B23 has been proposed to be a nuclear acceptor of the HIV-1 Rev protein. Both forms bind the Rev protein. Rev NLS peptides had a 5-fold greater affinity for B23 than SV40 T-antigen NLS peptides. In COS-7 cells expressing the Rev protein at relatively low levels B23 and Rev colocalized in the dense fibrillar and granular components of nucleoli. However, actinomycin D treatment reversibly released Rev and B23 from nucleoli suggesting that nucleolar localization of Rev is dependent on continued preribosomal RNA transcription. In cells with higher levels of Rev expression B23 and Rev colocalized at the perinuclear region. These data suggest that Rev interacts with B23 *in vivo*. Supported by NIH grants GM28349 and AI34277.

THE MA PROCEDURE IMPROVES THE VISUALIZATION OF NUCLEAR STRUCTURES

Mo - 160

FOR IMMUNOGOLD ASSAYS

P.S. Testillano^{1,2}, P. González-Melendi¹, J. Reyes,

K. Koberna, C.G. Mena¹, M.C. Risueño¹

¹Lab. Nuclear Organization in Plant Development, Centro Inv.Biológicas, CSIC. Madrid, Spain. ²Dep. CC. Morfológicas y Cirugía. Univ. Alcalá. Alcalá de Henares, Spain.

The methylation-acetylation (MA) method is an efficient blocking agent of proteins and, followed by uranyl or uranyl-lead staining, results in a preferential contrast of nucleic acid-containing structures. We have tested it on cryosections, and Lowicryl embedded plant and animal cells, and also the combination of this method with immunogold assays. When used before Lowicryl embedding, it provides a very good ultrastructural preservation and a much better visualization of the different nuclear structures. Moreover, it does not affect the main antigenic and chemical properties of the tissue and can be combined with immunogold labelling of DNA, RNA and various nuclear proteins, as well as with the BrdU method. Also, the sensitive TdT reaction method for DNA can be performed on MA treated samples. When ultrathin cryosections are treated with MA, an improvement in the contrast of chromatin is obtained, and the distinction between granular and fibrillar interchromatin structures is enhanced. The MA procedure is proposed as a simple cytochemical method for nucleic acids on immunogold studies of the nuclear organization in plant and animal cells.

We wish to thank Dr. Eilat and Dr. S.Muller for their kind gift of the anti-RNA and anti-histones antibodies. A part of this work was performed during a stay of K.Koberna (from Inst. Exp. Medicine, Prague) at the CIB, Madrid, supported by a Joint Research Program between CSIC and Czech Academy of Sciences. J. Reyes (from Centro Ingeniería Genética Y Biotec., La Habana, Cuba) is recipient of a grant from the ICI at the CIB, Madrid. Project CICYT PB 92-0079-C03-01.

AgNOR DISTRIBUTION IN ENDOMETRIAL
Mo - 161 CANCER

L.Buchinskaya, L.Polichshuk

Laboratory of Genetic, Institute of Experimental Pathology, Oncology and Radiobiology, Acad.Sci. of the Ukraine; Kiev, Ukraine

The argyrophilic nucleolar organizer regions /AgNOR/ and nucleoli morphological peculiarities of epithelial cells in patients with endometrial cancer and glandular hyperplasia have been studied on the cytological smears and histological materials. It had been shown the reliable increased number of AgNOR in tumour cells in comparison with in hyperplastic processes and a positive correlation with the level of tumours differentiation. The adenocarcinoma cells contain statistically significant high counts of nucleolonemic nucleoli and there are increasing level of large and small AgNOR's

in poorly differentiated cancer. Our data testify about diagnostic value of AgNOR and the possibility to use it as additional cytological parameter to determine the degree of endometrial cancer differentiation.

Development of in situ hybridization methods for the localization of RNAs at ultrastructural level.
Mo - 163 M.V.E. Macville^{a,b}, A.G.M. van Dorp^{a,b}, C.C. Wiesmeijer^{a,b}, J.A.M. Fransen^b, A.K. Raap^a.

Depts.^aCytochemistry & Cytometry and ^bElectron Microscopy, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. (NWO-MW no. 900-534-079)

In pre-sectioning electron microscopical ISH experiments, we studied the effect of various aldehyde fixatives and permeabilizing agents (such as organic solvents, proteinases and detergents) on signal intensity and ultrastructural morphology. In the rat 9G cell line, expressing the immediate early (IE) gene of human cytomegalovirus, 28S rRNA or IE mRNA was detected using digoxigenin labelled probes, followed by an immunoperoxidase/DAB reaction. Reflection contrast microscopical monitoring enabled us to detect low intensity ISH signals and predict subcellular morphology to a limited extend. EM examining of cross- and flat sections revealed ultrastructure and ISH detection efficiency. An acceptable balance between detection efficiency and ultrastructural preservation was found in 1% FA/0.05% GA fixed cells permeabilized with 0.1% saponin. 28S rRNA ISH signals were found throughout the cytoplasm associated with ribosomes, but not in nucleoli due to inaccessibility. Nevertheless, a large nuclear IE mRNA ISH signal was found close to the nuclear membrane and in smaller dots in the cytoplasm associated with RER and ribosomes. We conclude that with this method high and medium abundant RNAs can be detected in cultured cells with high efficiency at ultrastructural level without compromising morphology.

DOES THE W CHROMOSOME OF THE LEPIDOPTERAN *EPHESTIA* ENCODE A COMPONENT OF GERM PLASM ?
Mo - 162 CYTOLOGICAL PRESUMPTIONS.

M. Guelin

Laboratoire de Biologie cellulaire, Université Pierre et Marie Curie, 12, rue Cuvier, 75005 - PARIS, France.

In *Ephestia kuhniella*, in the heterogametic female sex (ZW), the W chromosome, with which no function is known to be associated, is, in whole or in part, maintained in heterochromatic state in interphase nuclei, mostly polyploid, of all cellular categories, except for the oocyte, at all stages of development. In nurse cells, however, for the whole length of the previtellogenesis, W-sex heterochromatin (W-SH) exhibits transcriptional activity. This phenomenon is accompanied by accumulation in the neighbouring cytoplasm of large masses of dense material usually described under the name of "nuage".

Analysis with ultrastructural cytochemistry (EDTA staining method and enzymatic extractions) and autoradiography (pulse-chase experiment using 5^3H) of the differentiated structures specifically associated, both in space and time, with active W-SH, leads to the hypothesis that the transcribed sequences correspond to one or several genes located within the heterochromatin of the W chromosome and escaping the global inactivation process. Our results suggest that W-SH transcripts : 1) are packaged within RNP poly particles typically resembling the hnRNP complexes isolated from the nuclei of numerous Eukaryotes; 2) are transferred to the cytoplasm through the nuclear pores; 3) accumulate upon leaving the nucleus within the nuage. Furthermore, the later, in addition to RNA, is shown to possess a high protein content and is likely to be an active seat of protein synthesis.

In *Drosophila*, the nuage has been proposed to be a site of assembly of germ plasm, then transferred to the oocyte. Application to *Ephestia* of this hypothesis, which is consistent with the plurimolecular composition of the nuage revealed in the present work, then leads to speculate that the putative W-SH gene(s) encode either a component of the germ plasm or some element involved in elaboration of the later.

Mo - 164 INTRANUCLEAR COMPARTMENTS EXPRESSING RNP AUTOANTIGENS

N. Ringertz, M. Wahren, I. Blange, W.-Q. Jiang and I. Pettersson
Dept. of Cell and Molecular Biology, Karolinska Institutet,
171 77 Stockholm, Sweden

Digital 3D immune fluorescence microscopy has been used to examine the intranuclear expression of RNP autoantigens Sm, RNP, Ro/SS-A, La/SS-B as well as antigens associated with EBV virus encoded transformation proteins (EBNA1-6) and tumor suppressor proteins (RB and p53). The Sm and RNP antigens show speckled immune fluorescence patterns corresponding to at least three distinct subcompartments, the "splicing islands", the coiled bodies and the microdots seen with the F78 MAB. The two proteins Ro60kD and Ro52kD associated with the cytoplasmic Ro/SS-A RNA-protein particles can also be detected in the nucleus. The Ro60kD nuclear immune fluorescence pattern overlaps with the "splicing island" fraction of the Sm-associated pattern. The domains expressing RNP antigens do not coincide with nuclear bodies containing viral transformation proteins and RB. Image analysis of immuno-fluorescence patterns suggest that the interphase nucleus is more highly organized than previously thought and that it contains several organelles or functional domains the function of which is not fully understood.

Mo-165 HETERO- AND HOMOLOGOUS CHROMOSOME ASSOCIATIONS IN DIFFERENT CELL POPULATIONS OF MOUSE AND CATTLE.

T.T.Glazko Laboratory for Molecular Genetics of Farm Animals, Institute of Animal Breeding and Genetics, v.Chubinski, Kiev-Borispol, 256319, Ukraine.

We investigated the specificities of chromosome interactions in some models: hetero- and homologous chromosome associations at the pericentromere regions and the homologous chromosome associations at the another chromosome regions in metaphase plates of the bone marrow cells, embryo fibroblasts cell lines of different mouse strains and the associations of sex chromosomes in the blood cells of cattle. The hetero- and homologous chromosome interactions (associations) in the mouse bone marrow cells and in the fibroblast lines at the different stage of the spontaneous neoplastic evolution were different and exhibited the cell line specificities. This associations were changed throughout the cell transitions from *in vivo* conditions to *in vitro*. The space closeness of homologous chromosomes at the metaphase plates (and, possible, in interphase nucleus) are high variability traits even in the cell populations of the same direction of cytodifferentiation and may be related with the another characteristics of karyotype instability, in particular, with aneuploidy.

REGULATION OF GLIAL FIBRILLARY ACIDIC PROTEIN EXPRESSION IN CELL TYPE OF NON-NEUROECTODERMAL Mo-167 ORIGIN

Laurence Lossouarn, Danielle Gomes, *Douglas Feinstein, Maria Galou and Pierre Dupouey

Unité de Biochimie des Antigènes, Institut Pasteur, 75015 Paris France and * Department of Neurobiology and Neuroscience, Cornell Medical Center, New-York, NY 10021, USA

Glial fibrillary acidic protein (GFAP), the major component of vertebrates astroglial intermediate filament, is also expressed in certain cell types outside the CNS. Each so far described extraneural expression of GFAP is specific of one tissue and one animal species. We are particularly interested in mouse lens epithelial cells (LEC) and hamster adrenocortical expression of GFAP.

We previously showed that among mouse species or hamster species, extra-neuronal GFAP expression revealed a polymorphism. Only certain species present extra-neuronal GFAP expression.

Here we show that DNA sequences directing transcription do not seem involved in permissivity of GFAP expression in extra-glial cells. So we investigate further in protein synthesis process.

We have used competitive polymerase chain reaction (PCR) technique to determine the levels of GFAP mRNA expression in mouse LEC and hamster adrenocortical cells from species that express or not the protein GFAP. The presence of GFAP mRNA was detected in LEC and adrenal gland of species that do not express the protein.

These studies confirm the expression of GFAP in non-neuronal tissues, and suggest that such ectopic expression may be controlled at a post-transcriptional level.

A NOVEL NUCLEAR PROTEIN OF 155kDa IS MODIFIED IN MITOSIS

Mo-166 A. Stachora, M. Pohlmeier and H. Ponstingl
German Cancer Research Center, Division Molecular Biology of Mitosis, D-69120 Heidelberg, FRG

Using a monoclonal antibody we have purified a nuclear protein from HeLa cells. Antibodies raised against this protein stain the cell nucleus with an increased signal in the nucleolar region. In preparations of HeLa nuclei the protein is found exclusively in the nuclear fraction, not in the cytoplasm. After disruption of the nuclei by sonication it remains in the insoluble fraction. From this fraction it can be extracted with high salt buffer, but not with detergents. The molecular weight of the protein is 155 kDa in SDS-PAGE, 340-360kDa in gel filtration and has a sedimentation coefficient of 7S in glycerol gradient centrifugation. This indicates an unusual structure or association with other cellular components. Comparison of partial amino acid sequence data to current data bases revealed no sequence homologies. To investigate the expression of this protein during the cell cycle we released HeLa cells from a double thymidine block and added nocodazole 8 hours after the release. We detected an upward shift in molecular weight of about 15kDa in SDS-PAGE correlating with G2/M as seen by comparison with DNA content and cdc2 kinase activity. This shift may be caused by phosphorylation. In indirect immunofluorescence studies we found that in mitosis the protein spreads over the whole cell except the chromosomes. After mitosis it relocates to the nucleus.

IDENTIFICATION AND QUANTIFICATION OF AG-NOR PROTEINS ON WESTERN BLOTS

Mo-168 P. Roussel^a, V. Sirri^{a,b} and D. Hernandez-Verdun^a

^a Institut Jacques Monod, 2 place Jussieu, 75251 Paris, France; ^b Dipartimento di Patologia Sperimentale, via San Giacomo 14, 40126 Bologna, Italy.

The ribosomal genes are associated with a set of silver-stained nucleolar proteins, the Ag-NOR proteins, whose amount is directly related to the duration of the cell cycle. We demonstrated that the detection of the Ag-NOR proteins can be performed on western blots^{1,2}. In the present work, a procedure has been established that allows quantification by densitometry of each Ag-NOR protein, using either purified proteins or a mixture of nuclear proteins. The major Ag-NOR proteins that are present during mitosis and interphase have been identified using 1-D and 2-D western blots, purified proteins and antibodies. During interphase, the major Ag-NOR proteins were nucleolin and protein B23 and also proteins of 42, 40 and 29 kDa, that accounted for a small amount of the silver stain. During mitosis, the major Ag-NOR proteins associated with the ribosomal genes were the largest RNA polymerase I subunit, the 135-kDa NOR protein, the UBF transcription factor and a 50-kDa protein. This study demonstrates that the major Ag-NOR proteins in nucleoli during interphase are not the same as those associated with the ribosomal genes during mitosis.

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Mo - 169

INTERRELATIONS OF PROSOMES WITH
INTERMEDIATE FILAMENTS, MICROFILAMENTS
AND ACTIN FIBERS IN EPITHELIAL,
FIBROBLASTIC AND MUSCLE CELLS

Arcangeletti C.^{1,2}, Missorini S.², Pinardi F.¹, Grand M.C.³, Vassy J.⁴,
Aebi U.⁵, Chezzi C.¹, Fouquer J.³ and Scherzer K.².
(1) Istituto di Microbiologia, Università degli Studi di Parma, Via Gramsci 14, 43100 Parma, Italy (2) Institut J. Monod, CNRS, Université Paris 7, 2, place Jussieu, 75251 Paris Cedex 05, France (3) UFR Biomédicale, Université Paris 13, 74, rue Marcel Cachin, 93012 Bobigny Cedex, France (4) URBB Unité INSERM U236, Université Paris 7, 2, Place Jussieu, 75251 Paris Cedex 05, France (5) Müller Institute, Biozentrum, University of Basel, Klingelbegstrasse 70, CH-4056 Basel, Switzerland.

It has been well known for many years that, in animal cells, mRNAs are bound to cytoskeletal structures resisting extraction by mild detergents such as Triton X-100. This allows to study the cytolocation of mRNA and mRNA-associated factors. Recently, it was proposed that actin filaments carry polyribosomes and, thus, actively translated mRNAs. Prosomes are sub-complexes of untranslated messenger ribonucleoproteins (mRNPs) having a proteinase activity (Multi-Catalytic Proteinase, MCP, or Proteasomes), and a putative regulatory function as trans-acting factors at the mRNA level. Triton-resistant prosomes were previously found associated primarily with the intermediate filaments (IFs) in epithelial, fibroblastic and muscle cells (Olink-Coux *et al.*, *J. Cell Sci.*, 1994, in press). Different fixation/extraction procedures allowed observation of a variable distribution of prosomes of specific subunit composition, among IFs and actin filaments, when using paraformaldehyde at 37°C in the presence of high Triton concentrations with PtK1 epithelial cells, Flow 2002 human fibroblasts and C2.7 myoblasts, or chilled acetone or methanol with skeletal muscle satellite cells. In contrast to the resistance of the prosome-IF interaction, a variable fraction of prosomes of specific subunit composition seems to be released from the actin filaments by Triton X-100 when applied prior to fixation. Moreover, *in vitro* copolymerisation of G-actin with prosome-like particles, isolated from neoplastic Hut-14 fibroblasts, allowed observation in the electron microscope of ladder-like filamentous structures where the prosome-like particles cross-linked actin filaments in a regular pattern. These results might indicate that prosomes of specific subunit composition, transiently associated with untranslated mRNA, are bound not only to IFs but also to actin filaments. When released with the other trans-acting factors from the mRNA prior to translation, they might somehow participate in the formation of actin cables (i.e. microfilaments and/or "stress fibers").

**ADP-RIBOSYLATION OF NUCLEOLAR
PROTEINS IN TUMOR CELLS**

Mo - 171 Jozefa Wesierska-Gadek and Norbert Leitinger
Institute of Tumorbiology-Cancer Research,
University of Vienna, Vienna, AUSTRIA

Modification of nucleolar proteins is of great interest because of their important function in transcription of rRNA genes and ribosomal biogenesis. ADP-ribosylation reactions in nucleoli of exponentially growing malignant cells were studied. In our first experimental approach, cellular proteins were *in vivo* labeled with ³⁵S-methionine and ADP-ribosylated proteins were isolated by affinity chromatography. In our next experiments nucleolar proteins were labeled with radioactive precursor ³²P-NAD. The nucleolar proteins were analysed by 1-dimensional and 2-dimensional polyacrylamide gel electrophoresis (PAGE) and the modified proteins detected by autoradiography. Electrophoretic analysis revealed that two characteristic nucleolar proteins at about 37 kD, pI 4.9-5.1 and at 100 kD, pI 5.4-5.6 were predominantly modified. The position of the radioactively labeled spots coincided with those characteristic for the major nucleolar phosphoprotein numatrin (B23) and nucleolin (C23). The identity of the modified nucleolar proteins was additionally proven in immunoblotting experiments using specific antibodies.

EXPRESSION OF HETEROLOGOUS HISTONE H1
IN YEAST

Mo - 170

G. Miloshev^a and J. Zlatanova^b

^aInstitute of Molecular Biology; ^bInstitute of Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria

Histone H1 binds to the linker DNA between nucleosomes and is involved in the formation and maintenance of higher order chromatin structure. It is believed that H1 inhibits transcription by as yet unknown mechanism. To elucidate the functions of this histone we introduced an foreign H1 histone gene in the yeast *S.cerevisiae*. The yeast *S.cerevisiae* was the obvious choice for such experiments, because of its simplicity and absence of a typical histone H1.

We report experiments on yeast transformation with expression vector carrying the gene for sea urchin histone H1 under the control of GAL inducible promoter of *S. cerevisiae*. Evidence that introduced gene is expressed to give the expected mRNA and protein products are presented. Some preliminary results of changes that occur with chromatin structure of yeast cells after histone H1 expression will be presented.

DYNAMICS OF PROSOMAL ANTIGEN CYTODISTRIBUTION
DURING *IN VITRO* DIFFERENTIATION OF SKELETAL

Mo - 172 J. Fouquer¹, M.C. Grand¹, S. Missorini², C. Arcangeletti³, K. Scherzer² and I. Martelly⁴
(1) UFR Biomédicale, Université Paris 13, 74, rue Marcel Cachin, 93012 Bobigny Cedex (2) Institut J. Monod CNRS Université Paris 7, 2, place Jussieu, 75251 Paris Cedex 05 (3) Istituto di Microbiologia, Università, Parma (4) URA CNRS 1813-MYREM Université Paris 12, Av. General De Gaulle, 94012 Créteil Cedex.

In skeletal and cardiac muscle, some prosome* (MCP/ proteasome) antigens were found to be interspersed into the sarcomeric pattern. On the other hand, colocalisation of prosome antigens with intermediate filaments (IF) of the various types, and, in particular, with desmin in C2.7 myoblasts was found previously**. The present study aimed to analyse the location of specific prosomes in course of myogenic differentiation in relation to the cytoskeleton. Therefore, we used *in vitro* primary cultures of satellite cells which are myogenic stem cells for muscle growth and post-traumatic muscle regeneration in adults. When grown *in vitro* after being dissociated from the muscle fibers, they differentiate into myotubes mimicking, thus, part of *in vivo* myogenesis. Monoclonal antibodies (mAbs) raised against a prosomal sub-unit (p27K) were used for immunolocalisation of prosomal particles in acetone fixed satellite cells at subsequent stages of differentiation. — Three days after plating, in proliferating cultures, the p27K antigen detected by IIF labeling was observed in a cytoplasmic, cone-like perinuclear area. In older cultures (days 4-5), the labeling was mainly found in the nucleus, where it appeared first in a granular, and later in a reticular pattern. Thereafter (days 7 to 10) in differentiating pre-fusion cultures, the labeling shifted from the nucleus to the cytoplasm where it formed a fibrillar pattern. In older cultures (from day 10 to 13) myotubes were formed. At this stage, only cytoplasmic labeling was observed. The cyt distribution of p27K progressively changed from a fibrillar pattern to an adult sarcomeric-like striation growing from the cell periphery. Using p27K mAbs in double-label IIF, colocalisation of prosome particles with the desmin-type IF and with actin filaments was observed. These results, showing a sequential nucleocytoplasmic traffic of prosomes during myogenesis and colocalisation with desmin and actin filaments emphasized the putative role of prosomes in intracellular mRNA addressing.

* Scherzer K. and Bey F. Prog. Nucl. Ac. Res. Mol. Biol. (in press).
** Olink-Coux, M. *et al.* *J. Cell Sci.* (1994) (in press).

Mo - 173 PROSOMES (MCP-PROTEASOMES) OF SPECIFIC SUBUNIT COMPOSITION ARE PRESENT ON THE INTERMEDIATE- AS WELL AS ON THE MICROFILAMENTS, AND ON THE TUBULIN NETWORK, IN RELATION TO THE PHYSIOLOGICAL STATE AND CELL CYCLE - K. SCHERRER^a, C. ARCANGELETTI^a, S.

MISSORINI^b and C. CHEZZI^c - Institut Jacques Monod, 2, Place Jussieu - Tour 43, 75251 PARIS Cedex 05, France (tel. 33 1 4427-6959 / fax -7647), ^aInstituto di Microbiologia, Università di Parma, Italy.

PROSOMES (PS) are ubiquitous small RNP particles first observed associated to untranslated mRNP (for review see 1). In higher eukaryotes, the PS particles (12 x 17 nm in the EM; MW 720.000) are composed of 26-28 proteins (20.000 - 36.000 MW) present in variable sets; a fraction of prosomes contains the "retroviral-type" tRNAly³, the reverse primer of HIV. About 50 Prosome-proteasome protein sequences from many species form various gene sub-families, all related phylogenetically to the two subunits of the α - and β -type in the archeobacterium *T. Acidophilum*. Biochemical and cytological data from the nematode to the human show that each cell and tissue has its characteristic set of PS with specific composition. Furthermore, some PS types are found in restricted, functionally relevant zones of the cytoplasm (cf. Grand et al, and Fouqueret et al, this Congress)

Serving as cytological markers for untranslated mRNA resisting Triton X-100 extraction, they were found, using specific monoclonal antibodies (mAbs), in part on the intermediate filaments (IF) of the various types, and on the microfilaments, in proportions varying with the antigen probed and the physiological state of the cells (cf. Arcangeletti et al, this Congress). They are also on chromosomes prior to and during transcription, on the nuclear matrix, as well as on lipo-soluble structures in the cytoplasm, at the surface of cells, and free in the blood serum. In mitosis, they are present on the mitotic spindle, on centrosomes and asters. - PS seem thus to relate to all kind of cytoskeletal elements in a cell function and stage-related manner, being possibly involved in nucleo-cytoplasmic mRNA transport.

(1) Scherrer K. and Bey F. Prog. Nucl. Ac. Res. Mol. Biol. (in press)

Mo - 175 CHARACTERIZATION OF SOME DNA-BINDING PROTEINS BY IMMUNOLOGICAL METHODS IN THE NUCLEAR MATRIX OF DINOFLAGELLATES. A. Minguez^a, S. Franca^b and S. Moreno Díaz de la Espina^a.

^aLab. Biología Celular y Molecular Vegetal. Centro Investigaciones Biológicas. CSIC. 28006 Madrid. Spain; ^bLaboratorio Microbiología Experimental. Instituto Nacional Saúde. Lisboa. Portugal.

Dinoflagellates are the only eukaryotic phylum lacking histones and nucleosomes. Sequence analysis of 5S RNA and snRNA localize the Dinoflagellates in evolution at the plant-animal divergence and suggest that the absence of histones and nucleosomes in them could be the result of a secondary loss and not a primitive character. To investigate the role of the Dinoflagellate nuclear matrix (NM) in organizing their non-nucleosomal DNA in structural and functional loop domains, its proteins were separated by SDS-PAGE and the presence of two nuclear matrix proteins involved in DNA binding (lamins and topoisomerase II) was investigated by immunoblotting and immunofluorescence labelling with a battery of specific antibodies. Three bands at 62, 58 and 50KD show crossreactivity with antibodies against both A and B type lamins, while the antitopo II reacts with a single band at 178 KD. The presence of lamins and topo II, both involved in the binding of MAR sequences to the NM in eukaryotes, suggests that the NM in Dinoflagellates plays a role in organizing functional DNA loops and that the two mechanisms of control of gene expression, nucleosomes and loop domains, are independent. Experiments in progress on the association of conserved MARs to these proteins, would clarify the functions of the NM in the organization of loop domains, and also allow evaluation of the conservation of the MAR sequences and the corresponding binding proteins along evolution.

Mo - 174 NUCLEAR ORGANIZATION AND ITS CORRELATION WITH RNA AND PROTEIN SYNTHESIS DURING EMBRYOGENIC INDUCTION IN POLLEN OF NICOTIANA TABACUM L.

M.I. Rodríguez-García^a, D. Garrido^{a,b}, O. Vicente^c, and E. Heberle-Bors^c

^aDept. de Bioquímica y Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín (C.S.I.C.) Prof. Albareda 1, 18008 Granada, Spain. ^bInst. de Biotecnología, Facultad de Ciencias, Universidad de Granada, 18001 Granada, Spain. ^cInst. of Microbiology and Genetics, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria.

The aim of this contribution is to report the behavior of vegetative and generative nuclei during the induction of pollen embryogenesis. We also determined RNA and protein synthesis during the induction period. By *in situ* hybridization using a ribosomal RNA probe 18S, we observed the pattern of ribosomal accumulation in grains freshly isolated from anthers and in embryogenic pollen.

The drop in RNA and protein synthesis during the embryogenic induction period is compatible with the results of *in situ* hybridization with regard to the differences found between freshly isolated young bicellular grain and embryogenic pollen. In embryogenic pollen, fewer gold particles were present in the nucleolus and cytoplasm. The considerable decrease in nucleolar volumen and structural organization, together with the progressive chromatin condensation and the loss of pores on the vegetative nuclear envelope are all signs of a decrease in nuclear activity during embryogenic pollen induction.

Supported by DGICYT Project n° PB92-0079-CO3-03 and Spanish-Austrian international cooperation.

Mo - 176 THE SPATIAL RELATION OF NUCLEAR RNA, SPLICING snRNPs AND CHROMOSOME DOMAINS PROVIDES EVIDENCE FOR AN INTER-CHROMOSOME DOMAIN COMPARTMENT

R.Zirbel, U.Mathieu, S.Lampel, A.Kurz and P.Lichter

Deutsches Krebsforschungszentrum, HEIDELBERG, Germany

Messenger-RNA, splicing snRNPs and chromosome domains represent nuclear entities, which can be visualized within distinct areas using fluorescence *in situ* hybridization and immunodetection procedures. The function of focal accumulations of splicing snRNPs (foci) is still under debate: they are considered to be sites of spliceosome assembly and/or storage or sites of active splicing. Following simultaneous visualization of snRNP foci and different nuclear RNAs, we found a co-localization of snRNP foci and transcripts of the heat-shock protein genes 84 and 86, but not of the foci and transcripts derived from EBV and HPV viral integrates. This indicates that splicing of pre-mRNAs is not restricted to the focal snRNP accumulations.

Since the DNA of an individual chromosome - composed of coding and non-coding regions - occurs within a distinct territory, we addressed the question, whether there is a non-homogeneous spatial distribution of transcription and RNA-processing within chromosome domains. Individual chromosome domains were visualized simultaneously with snRNP foci; moreover, chromosome 8 and the transcripts of HPV viral genes integrated into this chromosome were delineated differentially. Both, snRNP foci and HPV transcripts were found to be predominantly located in the periphery or outside of the territories occupied by individual chromosomes and excluded from their interior. Based on such findings, a model for the functional compartmentalization of the cell nucleus is presented. According to this model the space between chromosome domains, including their surface areas, defines a three-dimensional network-like compartment, termed the interchromosome domain (ICD) compartment, in which transcription and splicing of mRNA take place. Further details as well

TOWARDS A MOLECULAR UNDERSTANDING OF
NUCLEAR PORE COMPLEX (NPC) STRUCTURE AND
FUNCTION

Mo-177

N. Panté^a, R. Bastos^b, I. McMorrow^b, K.N. Goldie^a, B. Burke^b
and U. Aebi^a

^aM.E. Müller Institute, Biozentrum, University of Basel, Switzerland;
^bDepartment of Cell Biology, Harvard Medical School, Boston, USA.

The NPC is a ~120 MDa supramolecular assembly embedded in the double-membraned nuclear envelope (NE) that mediates nucleo-cytoplasmic transport in eukaryotic cells. Distinct peripheral NPC components such as the cytoplasmic and nuclear rings, the cytoplasmic filaments, and the nuclear baskets have been visualized by a number of different EM specimen preparation and imaging methods, and 3-D electron microscopy (EM) has revealed the basic design and architecture of the NPC (reviewed in [1, 2]). Thus far, the molecular architecture of the NPC has remained elusive, and no consensus has yet been reached on the central channel complex and some of the peripheral NPC components mentioned above. Using colloidal gold-tagged antibodies directed against a number of NPC proteins combined with quick freezing/freeze drying/rotary metal shadowing or embedding/thin sectioning, we have localized two NPC polypeptides to distinct peripheral NPC components. Labeling with three different antibodies has documented that NUP153 is a constituent of the terminal ring of the nuclear baskets, and p250 is a constituent of the cytoplasmic filaments. In addition, p250 was found in a complex of ~1.5 MDa with a novel NPC protein p75, which must also be located at the cytoplasmic face of the NPC. To investigate the presence and 3-D structure of the central channel complex in a more systematic way, we are testing a number of chemical fixation protocols and buffer conditions. Accordingly, NPCs lacking a central plug are yielded if exogenous ATP is present during specimen preparation, while ~95% of the NPCs harbor a pronounced central plug when NEs are treated with Cu-orthophenanthroline, an oxidizing agent inducing S-S bridge formation. To visualize the native cytoplasmic and nuclear NPC topography, we have imaged NE-bound NPCs in physiological buffer by scanning force microscopy (SFM). We are also using this approach in an attempt to directly correlate NPC structure with function.

References

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THE DISTRIBUTION OF RNA CONTAINING
STRUCTURES IN NUCLEOLI OF BLOOD
CELLS

K. Smetana

Institute of Hematology and Blood Transfusion
Prague, Czech Republic

The investigation of distribution of RNA containing structures in nucleoli of blood cells at the light microscopic level was facilitated by simple cytochemical procedures using buffered suitable basic dyes under standard defined conditions. Generally, early differentiation or maturation stages of blood cells are characterized by a relatively uniform distribution of nucleolar RNA. Such nucleoli are replaced by ring shaped nucleoli or micronucleoli with a characteristic distribution of RNA. The latter represent terminal stages of the nucleolar development in maturing blood cells in which the nucleolar RNA transcription is apparently inhibited. The RNA transcription in ring shaped nucleoli seems to be reversibly decreased. The incidence of these mentioned nucleolar types may be modified under pathological as well as experimental conditions and represents an useful marker of the proliferative or resting state of nucleolated blood cells including the leukemic ones. The electron microscopic distribution of RNA containing structures in addition provides their characteristic image possibly related to both RNA transcription as well as processing.

THE NUCLEAR ARCHITECTURE ASSOCIATED WITH RNA
SYNTHESIS AND PROCESSING

Mo-178

I. Raška, K. Koberna, M. Dundr, I. Melčák,
I. Srámková, J. Velický
Laboratory of Cell Biology, CzAcadSci,
Albertov 4, Prague, Czech Republic

We have mapped a number of important macromolecules such as RNA polymerase II, sn(o)RNAs and sn(o)RNPs, splicing factors, hnRNPs or poly(A) sequences in the interchromatin space of the cell nucleus. We have complemented this *in situ* picture by an extensive exploration of the method depicting active RNA synthesis based on the incorporation of bromouridine into RNA of permeabilized cells. Even though the result of the nonisotopic incorporation has largely depended on experimental conditions used (permeabilization conditions, composition of the transcription buffer, temperature of incubation etc.), extranucleolar transcription sites could be unambiguously mapped to the perichromatin region of the cell nucleus, implicating frequently RNP structures of granular appearance. These were, however, located outside of domains exhibiting highest snRNP label, i.e. primarily coiled bodies and clusters of interchromatin granules, and represented a new category of the nuclear architecture. Besides this category of structures, the existence of a number of genuine nuclear domains was demonstrated on the basis of highly differential contents(=label?) of macromolecules mentioned above. The absence of the label over some of the domains indicated their exclusion from the direct participation in RNA metabolism. Even though the labeling results did not provide an unambiguous interpretation, we associated RNA processing events with several such nuclear domains within the interchromatin space.

THE NUCLEOLUS: A Multifunctional Organelle?

Mo-180
R.L. Ochs, T.W. Stein, Jr., and E.M. Tan.
Autoimmune Disease Center, The Scripps
Research Institute, 10666 N. Torrey Pines Rd., La
Jolla, CA 92037, USA

The nucleolus has traditionally been thought of as monofunctional - its only function being the synthesis and packaging of preribosomal RNA (pre-rRNA) for cytoplasmic ribosomes. However, this concept has recently been challenged with new findings that the interphase nucleolus may contain centromeres/prekinetochores (Ochs and Press, *Exp. Cell Res.* 200:339-350, 1992) and coiled bodies (Ochs et al., *J. Cell Sci.* 107:385-399, 1994). Other indications of novel nucleolar functions include nucleolar localization of c-myc RNA transcripts (Bond and Wold, *Mol. Cell. Biol.* 13:3221-3230, 1993), and nucleolar localization of HIV viral proteins rev (Cochrane et al., *J. Virol.* 64:881-885, 1990) and tat (Siomi et al., *J. Virol.* 64:1803-1807, 1990) and the HTLV 1 viral protein rex (Nosaka et al., *PNAS* 86:9798-9802, 1989). Individually, the significance of these findings is not yet apparent, but taken together they may be indicative of a nucleolus that can subserve more than one function.

IDENTIFICATION OF ADP/ATP TRANSPORTER
GENE IN *Plasmodium falciparum*

Mo-181 Hatin I., Jambou R., Jauréguiberry G.
INSERM U13, 190 Bd. Mc Donald 75944 Paris
Cedex-France.

Plasmodium falciparum is an eukaryotic micro-aerobiosis parasite causing malaria. The energy requirements are essentially provided by glycolysis during its intraerythrocytic life. Two groups (Kanaani; Choi) have described an ATP transport between *P. falciparum* and the host erythrocyte via an ADP/ATP carrier, supposed to be on the plasmatic membrane. The biochemical properties of this translocator are similar to the transporter, that usually is in the inner membrane of mitochondria. By a direct molecular biological approach, using polymerase chain reaction with primers based on conserved stretches of sequence of ADP/ATP transporter, in accordance with the codon usage of the parasite genome, we have shown the presence of a gene coding for this transporter. Unexpectedly, a single nuclear gene was found by hybridization experiments. Moreover, we have located by electron microscopy, using an heterologous antibody, an ADP/ATP carrier in the inner mitochondrial membrane, but no label was seen on the parasite plasmatic membrane. The RNA detection by *in situ* hybridization has determined a growth regulated expression of this messenger. These studies point out the preferential transcription of the messenger coding for the ADP/ATP transporter, during schizontes stage through merozoites and rings stages. On going experiments are in progress in order to sequence the gene of this transporter and to study the translocator RNA expression during the life cycle of *P. falciparum*.

LOCALIZATION OF MAJOR mRNA PROTEINS
IN HUMAN DIPLOID SKIN FIBROBLASTS

N. Korneyeva, L. Ovchinnikov, L. Gavrilova

Institute of Protein Research, Russian Academy of Science,
142292 Pushchino, Moscow Region, Russia

Cytoplasmic messenger RNA molecules are found in eucaryotic cells in association with specific proteins to form RNA-protein complexes known as messenger ribonucleoprotein particles (mRNPs). The most prominent mRNA proteins of rabbit reticulocytes are the 50,000 and 78,000 mol wt proteins. p50 is a member of the Y-box binding protein family and affects the mRNA translation. p78 is known as a poly(A)-binding protein (PABP) associated with the 3'poly(A) tail of mRNA. It has been suggested that PABP may be involved in various aspects of mRNA metabolism including nucleoplasmic transport, cytoplasmic mRNA stability and translation, and may be involved in ribosome biogenesis. Indirect immunofluorescent microscopy was used to study the distribution of p50 and PABP in human diploid skin fibroblasts. We showed differences in p50 and PABP localizations in these cells. It was found that mouse polyclonal antiserum against p50 of rabbit reticulocytes reveals diffuse staining in the cytoplasm of the fibroblast. Antiserum against PABP of rabbit reticulocytes revealed not only diffuse staining within the cytoplasm, but compact stained structures in the nucleus - the nucleolus. The data on the PABP localization in the nucleolus, the site of the pre-rRNA synthesis are in agreement with our finding of high affinity of PABP to rRNA: PABP specifically bound to rRNA immobilized on the Sepharose and eluted under high salt conditions. We showed that rRNA is an effective competitor of poly(A) mRNA in PABP binding on nitrocellulose filters.

Mo-183 CONTROL OF HEME SYNTHESIS IN ERYTHROID CELLS:
THE SYNTHESIS OF ERYTHROID δ-AMINOLEVULINIC
ACID (ALA) SYNTHASE REQUIRES IRON

D. Vyoral^a, H.M. Schulman^a and P. Ponka^{a,b}

^aLady Davis Institute, Jewish General Hospital; ^bDepts. of Physiol. & Medicine, McGill Univ., Montreal, PQ, Canada.

Erythroid and ubiquitous forms of ALA synthase (ALAS), the first enzyme of heme synthesis, are encoded by distinct genes. The mRNA derived from the erythroid ALAS (e-ALAS) gene contains an Fe-responsive element (IRE) at its 5'UTR similar to that present in the 5'UTR of ferritin mRNA. Since this sequence is responsible for translational induction of ferritin by Fe, the availability of Fe may control e-ALAS expression in hemoglobin-synthesizing cells. In this study we examined e-ALAS formation in murine erythroleukemia (MEL) cells incubated without or with transferrin (Tf), the physiological Fe donor for erythroid cells. To measure e-ALAS production, the cells were metabolically labelled with [³⁵S]methionine, lysed, subjected to immunoprecipitation using a specific antiserum against recombinant murine e-ALAS (provided by Dr. M.W. Hentze), and samples were then analyzed by SDS-PAGE and autoradiography. As compared to untreated cells, 2-h incubation of DMSO-induced MEL cell with 10 μM Fe₂-Tf increased e-ALAS biosynthesis by 5-fold. In further experiments, ALA production was measured in MEL cells by incubating them with 2-¹⁴C-glycine in the presence of succinylacetone which blocks further metabolism of formed ¹⁴C-ALA by inhibiting ALA dehydratase. We showed that a preincubation of DMSO-induced MEL cells with desferrioxamine (Fe-chelator) decreased ¹⁴C-ALA formation to 10-20% of control level while the addition of 10 μM Fe₂-Tf increased ¹⁴C-ALA formation by about 3-fold. These results support the hypothesis that e-ALAS is translationally controlled by Fe.

Mo-184 DNA DEPENDENT RNA POLYMERASE I, II
AND III OF HUMAN PLACENTA NUCLEI

V.M. Sedova, V.I. Vorob'ev

Laboratory of Cell Biochemistry,
Institute of Cytology Russian
Academy of Sciences. St.Petersburg
194064, Russia

Multiple forms of DNA dependent RNA polymerases have been isolated from the human placenta nuclei. According to the order of elution from DEAE Sephadex A-25 column the types of enzyme are identified as RNA polymerase I, II and III. RNA polymerase III may be fractionated on two subtypes which are different in sedimentation behaviour in 17.5-35 % glycerol density gradient. Identified types of RNA polymerases display optimal enzymatic activity under different conditions in ionic strength and concentrations of bivalent cations, nucleoside-triphosphates and glycerol. Sensitivity to a-amanitine action is typical for multiple forms of RNA polymerases of mammals. All forms of RNA polymerase have multisubunit structure. The data obtained indicate that large subunit with molecular weight about 220 kDa is common for identified forms of RNA polymerases from human placenta nuclei.

Mo-185 A SPECIFIC CLASS OF snRNPs CONTAINING RETROPOSONE-LIKE TRANSCRIPTS IN DIFFERENTIATED CELLS

I.M.Konstantinova, O.A.Petukhova,
L.V.Turoverova, V.A.Kulichkova,
I.V.Volkova, O.R.Ilkaeva and
I.V.Kozuharova

Institute of Cytology, RAS, Tikhoretsky
av., 4, St.-Petersburg, 194064 Russia

We have identified the specific small ribonucleoprotein particles (α -RNP) which contain specific set of heterogeneous small RNA (70-120 nt.) complexed to several proteins, main components in 20000-48000 mol. wt. range. The α -RNA are homologous to the repetitive elements of the SINE class (ID,B1,B2 in rat cells and ALU in human cells). The α -RNA molecules are transcribed by RNA polymerase III. α -RNA are capable of stable hybridization with specific messenger RNAs. The α -RNA expression is specifically regulated under gene regulatory stimuli. The gel mobility shift assay studies show specific interaction of the α -RNP with the ALU repeat DNA. The data obtained allow to suggest the α -RNP involvement in the coordinated regulation of the expression of the groups of genes, containing retroposone-like sequences in their regulatory regions.

Mo-186 VIRUS-INDUCED MODULATION OF PROTEIN BIOSYNTHESIS IN SENSITIVE AND RESISTANT PEA PLANTS

K.Ostrówka and I.Frenzel
Institute of Plant Genetics, Polish Academy of Sciences, 60-479 Poznań, Poland

Two approaches were undertaken using in vitro translation reaction towards recognition of mechanisms responsible for regulation of viral infection in pea (*Pisum sativum* L.) plants.

A competitive reaction of protein biosynthesis was performed, where poly(A)*RNAs of sensitive and resistant pea varieties were translated in the presence of RNA of two pathogenically different BYMV strains: one mild and one severe. The severe BYMV strain RNA caused a decrease of the quantitative level of protein synthesis of sensitive pea variety, whereas the amount of peptides of the resistant variety remained unaffected. The mild BYMV strain RNA did not influence the translation efficiency of mRNAs of any pea variety tested.

Poly(A)*RNAs were isolated from pea plants previously inoculated with two strains of PSV, mild and severe one. While translated in vitro, the mRNA obtained from plants infected with severe PSV strain revealed much lower efficiency of protein synthesis, as compared to those isolated from plants inoculated with the mild PSV strain.

The obtained results are discussed in relation to hypothesis of the translation process being involved in the regulation of plant response to viral infection.

RNA EDITING PATTERNS OF TRYPANOPLASMA BORRELI (KINETOPLASTIDA)

Mo-187 J.Lukeš^a, J.Van den Burg^b, G.J.Arts^b, A.de Haan^b, F.Opperdoes^c, H.Van Steeg^d, P.Sloof^b, R.Benne^b

^a Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic, ^b E.C. Slater Institute, University of Amsterdam, Amsterdam, The Netherlands, ^c Institute of Cellular and Molecular Pathology, Brussels, Belgium, ^d National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

We have studied *T. borreli*, a parasitic bodonid, phylogenetically distant from trypanosomatids hitherto studied. We have sequenced a 5.3 kb fragment of mitochondrial DNA and have found genes encoding cytochrome oxidase subunits I (cox I) and II (cox II), and apocytochrome b (CYb), which were arranged in a novel order when compared with other kinetoplastids. The analysis of transcripts of these genes revealed unexpected editing patterns. The genomic sequence of cox II predict lack of editing in the mRNA. At the 5' and 3' ends the transcripts of cox I and CYb, undergo extensive editing. Moreover, editing of cox I mRNA suggest the N' terminal aminoacid sequence of the protein being almost identical with the same region of *Trypanosoma brucei* protein, the transcripts of which, however, lack editing. The possible reverse transcription events in the evolutionary history of kinetoplastids are discussed.

Mo-188 ULTRASTRUCTURE AND FUNCTIONAL ACTIVITY OF THE CENTROSOME AFTER UV MICROIRRADIATION

R.E.Uzbekov, A.L.Neverova,
and I.A.Vorobjev

A.N.Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow, Russia.

Ultraviolet microirradiation (UV MI) of the PK cells centrosome ($\lambda_{max}=280\text{nm}$, diameter of the spot $1.6\text{ }\mu\text{m}$) at early anaphase slows down and then stops chromosome motion towards the irradiated pole. This happens as a result of rapid (in 2-3 min) disorganization of the halfspindle. Chromosome movement towards the opposite pole continues normally. Cell spreading after cytokinesis occurred normally, but then the cell with irradiated centrosome stop growing. Two hours after mitosis in the irradiated cell the amount of MT is less than in the sister cell, and MT radiating from the centrosome are practically absent. These cells didn't enter S-phase as long as for 24 hrs after division, while sister cells did. RNA synthesis in cells with irradiated centrosome was less, than in their sister cells. We conclude that UV irradiation of centrosome inactivate it and prevents formation of the radial MT array.

Centrosome irradiation affect on spindle immediately and irreversibly inhibit cell growth and proliferation.

Mo-189 MITOCHONDRIA CONVERT INTO POST-MITOCHONDRIA AND MICROORGANISMS

A.Belyakovich

ITEB, Pushchino, Moscow region,
142292, Russia

Using p-NTV as a vital dye of mitochondria and bacteria it was shown that in intact cells mitochondria convert into postmitochondria and in destroyed cells mitochondria convert into microorganisms (1,2).

Two groups of mitochondria ("big" and "small") were observed with p-NTV in intact cells. Big mitochondria appear, live and disappear by groups, following one group after another as waves. When mitochondria of the group convert into postmitochondria there appear another group of organelles. Author could not convert mitochondria into postmitochondria *in vitro*.

In destroyed cells mitochondria, repeating character of mitochondria-postmitochondria conversion, convert into microorganisms with DNA of 16-20 kb (bacterial DNA range 800-8000 kb). In some media microorganisms are able to move and propagate. All observations (2) allowed author to conclude that life is a result of interaction of genome and medium.

1. A.Belyakovich. Mitochondria convert into microorganisms ?! Preprint. Pushchino, 1989, 28 pp.

2. A.Belyakovich. Study of mitochondria and bacteria using tetrazolium salt p-NTV. Pushchino 1990, 233 pp.

UNUSUAL MITOCHONDRIA REPRODUCTION

Mo-190 IN CULTURED PLANT CELLS

D.A. Klimchuk

Istitute of Botany, Academy of Sciences, 252004 Kiev, Ukraine

Unusual structures (US) of mitochondria related to their reproduction were found in pea cells *in vitro*.

Tissue culture has been obtained from *Pisum sativum* L. seedling roots and it has been grown in darkness at 25°C on the Murashige and Scog modified agar medium.

In the three-dimensional reconstruction US are flat cisterne of either oval or round shape with 3-5 mkm in diameter and the width 0.1-0.2 mkm - in the central area and 0.4 - 0.6 mkm - on peripheric area. They are surrounded by an outer and an inner membrane. US has dumb-bell-like shape on the micrograph tangential sections. Membrane complex (MC) of 30-50 nm in the width are located in the central area of US. The MC has high electron density and consists of 3-5 closely packed membranes. The space between the envelope and MC has structural organization typical for matrix of mitochondria which separate from peripheric area of the US. The US are observed in cytoplasm of meristem-like cells in logarithmic phase of culture growth. The function of the US may be connected with very rapid reproduction of the organelles in the period of cell active growth.

RAT LIVER MITOCHONDRIAL PROCESSING PEPTIDASE (MPP): EXPRESSION IN *E.COLI* AND SITE DIRECTED MUTAGENESIS OF ITS β -SUBUNIT

P.Rysavy, H.-M.Striebel and F.Kalousek. Department of Genetics, Yale University School of Medicine, New Haven, CT 06510 USA

Nuclearly encoded mitochondrial matrix proteins are synthesized with a leader peptide that in the majority of cases, is cleaved after import in one step by mitochondrial processing peptidase (MPP). The enzyme, isolated and purified from rat liver mitochondria, consists of two nonidentical subunits (α (55kDa) and (50kDa)) and forms a stable heterodimer complex. Both subunits contain a region of negatively charged amino acids which is predicted to form an amphiphilic α -helix that might function as a binding site for positively charged cleavable leader sequences. Moreover, the β -subunit has an inverted Zn^{2+} binding motif found also in *E.coli* protease III. We overexpressed the mature sequence of the α - and β - subunit in *E.coli* first as a fusion protein and recently as free subunits, and we showed that both subunits are necessary for the activity. The efficiency of the expression could be increased by the coexpression of bacterial chaperonins GroEL/GroES. To better understand the role of particular amino acid residues in MPP, we first focused our interest on the negatively charged region of β -MPP. We extended the polypeptide chain between amino acids 177-178 with alanine or isoleucine or exchanged glutamate 174 for aspartate. All three mutations resulted in a decrease of activity, but only extension with alanine caused complete loss of the enzymatic activity, which could be restored by adding wild type β -subunit. Supported by NIH grant DK 09527.

CELL CYCLE DISTRIBUTION OF A CENTROSOMAL ANTIGEN HIGHER PLANT CELLS

Mo-192

A.C. Schmit^a, V. Chevrier^b, D. Jobb and A.M. Lamberta

^a Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, UPR 406, 12 rue du Général Zimmer, F-67084 STRASBOURG-Cedex, France; ^bDépartement de Biologie Moléculaire et Structurale, Laboratoire du cytosquelette, INSERM U 244, Centre d'Etudes Nucléaires de Grenoble, 85 X, F-38041 GRENOBLE-Cedex, France.

Compelling evidence has been obtained in favour of the idea that the nuclear surface of higher plant cells is a microtubule-nucleating and/or organizing site (MTOC), in the absence of defined centrosomes. How these plant MTOC proteins are redistributed and function during the progression of the cell-cycle remains entirely unknown. Using a monoclonal antibody (mAb 6C6) raised against isolated calf thymus centrosomes, we followed the targeted antigen distribution during mitosis and meiosis of higher plants. During interphase and prophase, the protein is specifically detected at the surface of the nuclei. When the nuclear envelope breaks down, the antigen suddenly becomes associated with the centromere-kinetochore until late anaphase. In telophase, when the nuclear envelope is being reconstructed, the protein is no longer detected at the kinetochores but is solely associated again to the nuclear periphery. This protein displays a unique spatial and temporal distribution which may reflect the pathway of plant antigen(s) between the nuclear surface and the kinetochores under cell-cycle control. These observations shed light on the molecular organization of the plant kinetochore and particularly on its putative activity as a protein transporter. They also suggest that a plant centrosomal-like antigen may have different microtubule related functions depending on cell-cycle regulated changes in its subcellular distribution.

CENTROSOME AND MICROTUBULES IN THE CYTOPLASTS OF CULTURED CELLS

Mo - 193

L.A. Gorgidze, I.A. Vorobjev

A.N.Belozersky Institute of Physico-Chemical Biology
Moscow State University, 119899 Moscow, Russia

After enucleation of cultured cells using cytochalasin D centrioles remained approximately in 80% of cytoplasts from PK (pig kidney embryo) cells and in all cytoplasts from L-fibroblasts. Cytoplasts survive for 20-24 hrs keeping normal morphology, pattern of energized mitochondria and microtubules. Microtubules in cytoplasts became much more resistant to nocodazole treatment as compared with those of whole cells. Stable microtubules in cytoplasts emanated from the centrosome or were scattered at the periphery of the cytoplasm.

Centrioles in cytoplasts were able to turn perpendicularly to the substrate surface in response to ouabain treatment, as described previously for whole cells. This reaction is thus independent of the nucleus.

16 hrs after enucleation procentrioles were observed in some cytoplasts of PK cells. In the cytoplasts obtained from synchronized culture of L-cells during G1-period replication of centrioles took place at approximately the same time when it happened in normal cells. Actinomycin D when added in the late G1 inhibited centriole replication in nucleated cells, but not in cytoplasts. The results obtained show that nucleus regulate centriole replication both positive and negative. We suggest negative regulation of centriole replication to be accomplished through the nucleus-cytoplasmic transport.

MORPHOGENESIS OF CENTRIOLES AND CENTROSOMES IN MICE DEVELOPMENT

Mo - 194

S.S.Abumuslimov, E.S.Nadezhina
and Yu.S.Chentsov

A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow

To study morphogenesis of centrioles and centrosomes in mice development we used monoclonal antibodies RN-C6 against centrosomal phosphoproteins involved in centrosome induced microtubules polymerization. This monAb immunostained in 2-16 cell embryos the sperm tail only and nothing in 4.0 day blastula. RN-C6 immunostained small dots (centrosomes) in several interphase and mitotic cells of 4.5 day blastula and in many cells of the late blastula. We also studied mice embryos by electron microscopic serial ultrathin sections. We have found centrioles both in trophoectoderm and internal cellular mass cells in 4.0 day blastula. These centrioles were not connected with microtubules and had juvenile structure with distinct cartwheel. In 4.75 day blastula many cells had centrioles, and often they were surrounded by microtubule-organizing centres and microtubules. So, morphogenesis of centrioles *de novo* does not depend on cellular microtubules and goes from procentrioles to centrioles and then to centrosomes as microtubule-organizing centres.

ANTIBODIES SPECIFIC TO
PICORNAVIRAL RNA-VPg STAIN
CENTRIOLES

Mo - 195 E.S.Nadezhina^a, T.Ya.Demyanenko^b,
E.N.Baranova^b, V.P.Veiko^c,
Yu.F.Drygin^c

^aInstitute of Protein Research RAN, Pushchino,
Moscow Region, Russia; ^bA.N.Belozersky Institute
of Physico-Chemical Biology, MSU, Moscow,
Russia; ^cInstitute of Genetics and Selection
of Industrial Microorganisms, Moscow, Russia

Antiserum against encephalomyocardial viral (EMCV) RNA-VPg complex was obtained from immunized BALB/c mice. To prevent lethal infection of mice EMCV RNA-VPg was split off by RNase H in presence of oligo-dG and was then coupled with BSA by glutardialdehyde treatment. L cells infected with EMC virus were displayed by immunofluorescent microscopy using the serum. Surprisingly, centrioles of non-infected L cells or HeLa cells were stained with the serum. RNase treatment of cells or preincubation of serum with Tyr-(P->O)-pT₁₅ (synthetic model of RNA-VPg) diminished immunostaining of centrioles. Dot-ELISA showed preferential binding of antibodies obtained to RNA component of RNA-VPg complex. Isolation and study of centriolar RNA is in progress.

A NEW TYPE OF GTP-BINDING PROTEIN
OF THE CHLOROPLAST OUTER ENVELOPE

Mo - 196 M. Seedorf and J. Soll
Botanisches Institut
Universität Kiel
24098 Kiel, Germany

Most of the chloroplast localized polypeptides are nuclear coded and imported into the organelle from the cytosol in a posttranslational process. We strongly assumed that OEP 34 from the outer envelope of pea chloroplasts is a putative member of the plastidial protein translocation machinery. Using peptide derived degenerated oligonucleotide probes we have isolated a cDNA-clone for pea OEP 34. The deduced amino acid sequence revealed conserved sequence motifs found in GTP-binding proteins, making it a new member of this superfamily. OEP 34 was specifically photoaffinity labelled by [λ 32P] GTP in isolated outer envelope membranes. The obtained photoaffinity labelling occurred in a GTP dependent manner, i. e. the incorporation of the ligand was not decreased by the addition of one hundred fold excess of other nucleotides like ATP, CTP or GMP. OEP 34 is an integral part of the outer envelope membrane. Digestion with several proteases revealed a 7 kD protected degradation product which only disappeared in the presence of detergent. The preponderant GTP binding part of OEP 34 was protease sensitive, that means it is exposed to the cytoplasm. Targeting to chloroplasts requires an intact C-terminus of OEP 34 and the presence of protease sensitive chloroplast surface components.

**GENERATION AND PRELIMINARY
CHARACTERIZATION OF ANTI-TONOPLAST
MONOCLONAL ANTIBODIES**

Mo-197 P. Dozolme, D. Marty-Mazars, M-C. Clemencet and
F. Marty

Laboratoire de Phyto-Biologie Cellulaire, UA 692, Université de Bourgogne, 21004 DIJON Cedex, FRANCE.

In order to identify the many antigens which are present in the membranes of the vacuolar system in plants, specific monoclonal antibodies (mAbs) have been generated.

BALB/c mice were immunized intraperitoneally with a whole tonoplast (vacuole membrane)-enriched fraction from *Brassica oleracea* L. This approach resulted in the generation of murine monoclonal antibodies that specifically react with epitopes that are present on the surface of the vacuole membrane. At an early stage in the procedure, suitable clones were detected in the panel of hybridomas produced. Stable clones, fast growing, and secreting large quantities of antibodies with high affinity for the membrane were selected. The isotypes have been tested. All mAbs were shown by immunofluorescence microscopy to be highly specific for the vacuole membrane. Because a complex mixture of membrane antigens are present, we determined the mAbs specificity by Western-blot analysis. Despite some redundancy among the recognized proteins, several different tonoplast proteins with Mr ranging from 16,000 to 110,000 daltons were labeled. Although many proteins from the vacuole membrane were glycosylated, most of the epitopes were not carbohydrates, and they were specific for single polypeptides.

Further work will be needed to characterize the function of the proteins labeled by the mAbs.

**ARITHMETICAL DISSECTION OF ORGANELLE
TRAJECTORIES INTO MOVEMENT PATTERNS.**

Mo-198

A.H.N. de Win and J. Derksen.

Department of Experimental Botany, Catholic University Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, the Netherlands.

The movements of organelles in plant cells, previously called cytoplasmic streaming, are highly individual. Though descriptions of the types of movement have been made, quantitative data are rare. To quantitatively analyze organelle movements, the VIDAS image analysis system (Zeiss/Kontron) was used (1). Pollen tubes of *Nicotiana tabacum* were recorded on videotape and converted to digital image time lapse series. Particle centre's were indicated in the pixel field of the computer. The pixel coordinates were used to calculate the position in the cell, together with the distance, angle and velocity of each displacement.

The trajectories of the organelles were divided in stretches of directed or undirected movement using the SAS system. Directed movement was defined as a series of at least three displacements with a minimum length of 0,5 μm and absence of gross changes in movement direction. All other cases were characterised as undirected. A gross change in movement was detected when the x-coordinate was reversed or the movement angle had changed more than 90 degrees. Directional movement was characterised by the linearity ratio. Significant differences could be detected in directed and undirected movements in different parts of the tube, both in velocity and in number. These quantitative data could provide a more detailed insight into the mechanism of organelle movement.

(1) de Win, A.H.N., Derksen, J. and Lichtscheidl, I.K., 1993. Eur. J. Cell Biol. 60(s37):104.

**Mo-199 DO PLANT MITOCHONDRIA CONTROL PROTEIN
IMPORT THEMSELVES?**

U. Witt and W.O. Abel

Institute of Botany, Department of Genetics,
Ohnhorststr. 18, 22609 Hamburg, FRG

Malate dehydrogenase (MDH, EC 1.1.1.37), an NAD⁺-dependent dehydrogenase, occurs in multiple isomeric forms located in different cell compartments in plant tissues. The mitochondrial malate dehydrogenase (m-MDH) catalyzes the oxidation of malate to oxalacetate and is therefore a key enzyme of energy conversion in mitochondria. Various isozymes for m-MDH have been found in different mitochondria. They are nuclear encoded and imported into the mitochondrial matrix. This protein targeting into mitochondria is poorly understood in plant mitochondria but first results indicate differences to the import mechanisms of *Saccharomyces* and *Neurospora*.

Therefore we will report on the influence of different nuclear-mitochondrial combinations with respect to resulting m-MDH patterns in the mitochondria of *Brassica napus*. Different m-MDH patterns of this nuclear encoded enzyme are observed in different types of mitochondria isolated from isogenic plants, indicating that mitochondria themselves are able to control proteinimport.

**IDENTIFICATION AND CHARACTERIZATION OF
A NEW COMPONENT OF THE CENTROMERE-KINETO-**

Mo-200

M. Knehr, M. Demel, D. Schroeter,

U.-L. Kiesewetter, E.-M. Finze, N. Paweletz^a,
^aGerman Cancer Research Center, Dept. 430,
D-69120 Heidelberg, FRG.

Investigating different sera from patients with autoimmune diseases, serum SL allowed the identification of a new highly specific 58 kDa protein in WESTERN blotting of chromosomes and nuclei from HeLa cells. Light- and electron microscopy demonstrated a strong immune reaction with the centromere-/ kinetochore complex and some component of the ER beginning with prometaphase until telophase. Testing various antibodies revealed no relation to already known mitotic or centromeric proteins (CENPs). 2D electrophoresis of whole cell extract, nuclei and chromosomes followed by immune reaction resulted in two neighbouring dots with a pI of ~5.0. Interestingly, whole mitotic cells revealed additional dots at lower molecular weights.

Currently, we are determining cDNA and protein sequences by screening expression libraries of HeLa cell origin with our highly specific serum. This will allow further characterization concerning structural and possibly functional properties of the new CENP.

Mo - 201 Cytosolic Factors Involved In The Process Of Mitochondrial Protein Import.
Erik M. von Stedingk, Ulrika Fristedt, Marie Hugosson, Cristina Szigyarto, Elzbieta Glaser

A wide range of proteins with chaperone function are involved in the mitochondrial protein import: 1) Cytosolic chaperones and possibly other factors present the protein in an import-competent conformation to the import receptors; 2) Mitochondrial matrix-located chaperones are believed to play an active role in the actual protein translocation process; 3) Another kind of chaperones are involved in correct folding and transport of proteins to their assigned location within the organelle. We have studied the effect of the isolated cytosol on *in vitro* protein import in plants. We have used the *in vitro* translated precursor of the ATP synthase F1 β subunit from *N. plumbaginifolia*, synthesised in rabbit reticulocyte lysate, and isolated spinach mitochondria and cytosol. We report presence of cytosolic factors capable of altering the efficiency of protein import into leaf mitochondria from *Spinacia oleracea*. When mitochondria were preincubated with cytosol, before addition of the precursor protein, import efficiency was enhanced several fold. On the other hand, preincubation of the precursor with cytosol, before addition of mitochondria, impaired the import efficiency. Fractionation of cytosol and characterization of the cytosolic factors responsible for the observed effects are in progress.

Mo - 203 CENTRIN-RELATED PROTEINS IN ISOLATED HUMAN CENTROSOMES
A.Joly, M.Moudjou, N.Paintrand, J.L.Salisbury*, M.Bornens
CCM, CNRS, 91198 Gif, France
***Mayo Clinic, Rochester, MN 55905 USA**

Centrosome biogenesis and inheritance are still poorly understood issues in cell biology. One may assume that the centrosome duplication pathway is important enough for cell survival that it may have been conserved throughout evolution of eukaryotic cells and speculate that a relatively small number of essential genes may be involved in this pathway. Thus one could benefit from the particular experimental approaches which have been developed in each case, to get to general principle of centrosome inheritance.

As a matter of fact, striking conservation of several centrosomal proteins have been reported in widely divergent species from algae to mammals. For example, highly enriched centrosomes preparations isolated from human cells have demonstrated a complex protein pattern in which an insoluble calcium-binding protein with app. MW 62/64kDa was identified using a rabbit polyclonal serum raised against centrin from *Tetraselmis s.* (serum 08/28) and a rabbit serum raised against a 230kDa protein from *Parplastron m.* microfibrillar lattice (1). Interestingly, these antibodies efficiently blocked nucleation of microtubules, from PC-jubulin or in Xenopus egg extracts, on isolated centrosomes. None of these sera reacted with low molecular weight centrosomal components in human cells.

Two cDNAs have been recently cloned from human libraries encoding small calcium-binding proteins of about 20kDa with very high identity with Chlamydomonas centrin (2, 3). These proteins are apparently located to the centrosomes in human cells.

These results prompted us to re-investigate the possibility that the human highly conserved centrin-like proteins were overlooked in our previous work due to technical problems (low molecular weight proteins can easily diffuse from nitrocellulose filters).

We therefore undertook thorough comparison of the results obtained on human centrosomes with serum 08/28 with those obtained using a monoclonal antibody (20H5). Both antibodies give identical results on Chlamydomonas cells: they react with the 20kDa centrin and decorate the nucleo-basal body apparatus in a similar way. We affinity-purified IgG from serum 08/28 on Chlamydomonas centrin and used them, together with mAb 20H5, on human centrosomal proteins which were chemically cross-linked to the nitrocellulose filter after transfer. Non-overlapping results were obtained: only p62/64 was detected with 08/28 IgG whereas 20H5 failed to recognize the p62/64 protein but recognized two bands at about 20kDa.

Double immunofluorescence on human cells, and on isolated centrosomes, revealed a strikingly different decoration of the centrosome, 20H5 decoration being much more restricted to the centrioles. Accordingly, post-embedding ultrastructural studies on isolated centrosomes with 20H5 revealed that the 20kDa proteins were mainly concentrated within the distal lumen of the centrioles. Also, p62/64 and the 20kDa centrin behaved differently when centrosomes were subfractionated with various procedures. For example, the 20kDa centrins were largely solubilized by a 45°C treatment of isolated centrosomes during which p62/64 remained entirely insoluble. Finally, in contrast with serum 08/28, 20H5 did not block the nucleation of microtubules on isolated centrosomes in Xenopus oocyte extracts.

Only the molecular characterisation of the human centrosomal p62/64 will tell us if this calcium-binding protein involved in the microtubule nucleation reaction is evolutionarily related to centrin.

References: (1) Moudjou et al., J. Cell. Biol., 1991, vol 115, 129-140. (2) Errabolu et al., J. Cell. Science, vol 107, 9-16. (3) Huang et al., P.N.A.S., vol 90, 11039-11043

Mo - 202 MICROTUBULE BINDING ACTIVITY OF THE *S. cerevisiae* KINETOCHEORE
F.F. Severin*, P.K. Sorger^b & A.A. Hyman^a
^aEMBL, Heidelberg, Germany, ^bMIT, Boston, MA

Kinetochore proteins bind to centromere DNA to mediate the interaction of chromosomes with microtubules during mitosis. In *S. cerevisiae* the CBF3 complex links microtubules to centromere DNA in vitro. We have investigated which components of the CBF3 complex are responsible for microtubule binding. To assay microtubule binding activity we used beads with attached centromere DNA. After preincubation with yeast crude extract, these beads bound to microtubules attached to a coverslip. Extracts made from mutant yeast lacking one of the components of CBF3 complex did not support binding of the beads. Partially purified CBF3 complex could bind DNA but was greatly reduced in microtubule binding activity. When partially purified CBF3 was added to the extract of the mutant yeast lacking DNA binding, complete reconstitution of microtubule binding activity took place. It was found also that beads preincubated with the extract and then washed several times with buffer still retain microtubule binding activity. When 250 mM KCl was added to the wash buffer, the beads lost the microtubule binding activity and the addition of the mutant extract to such beads also lead to the reconstitution of microtubule binding activity. These data indicate that proteins other than those required for DNA binding are necessary for microtubule binding of the CBF3 complex. We found that these microtubule binding component of the kinetochore complex can be separated from the DNA binding component by gel filtration of an yeast extract.

We also found that the addition of ATP during the preincubation of the beads with the extract reduces their binding to microtubules. It appeared to be that microcystin, an inhibitor of protein phosphatases PP1 and PP2A, significantly enhances the effect of ATP, suggesting that kinetochore-microtubule interaction is regulated by phosphorylation.

Mo - 204 INTRA-SARCOMERIC CYTOLOCALISATION OF PROSOME ANTIGENS IN VERTEBRATE MUSCLE

M.C. Grand¹, F. Pinardi², J. Gautron³, C. Chezzi⁴, K. Scherrer² and J. Foucquier¹
(1) UFR Biomédicale, Université Paris 13, 74, rue Marcel Cachin, 93012 Bobigny Cedex. (2) Institut Jacques Monod CNRS / Université Paris 7, 2, place Jussieu, 75251 Paris Cedex 05. (3) URA CNRS 1813-MYREM / Université Paris 12, Av. du Général De Gaulle, 94012 Créteil Cedex. (4) Istituto di Microbiologia, Università di Parma, Italia.

Prosomes (MCP/Proteasomes) are "facultative" ribonucleoprotein complexes considered as cellular factors having a putative role in the homeostasis of specific cell proteins*. Indeed, prosomes might serve i) in post-transcriptional processing, in transport, distribution and control of stored mRNPs, including cytodistribution of specific mRNAs on the cytoskeleton, ii) in the processing and/or degradation of proteins corresponding to their multicatalytic proteinase activity. In view of their morpho-functional compartmentation related to the sarcomeric cytoplasmic organization, the muscle system represents a useful biological model to specify some biological functions of prosomes. Interestingly, in cultured myoblasts prosomes were found to colocalize with the desmin fibers of the cytoskeleton and with actin filaments. Therefore, the presence of prosome antigens was studied in skeletal and cardiac muscle, using monoclonal antibodies raised against several different prosomal sub-unit proteins. In view of its important labeling intensity, the cytolocation of the p27K antigen was particularly well analysed. A reproducible intra-sarcomeric distribution of p27K was observed in rat skeletal and cardiac muscle. In frog, only the skeletal muscle tissue showed this type of labeling. By double-label indirect immunolocalisation, the distribution of p27K antigen relative to actin and desmin was analysed. Moreover, using specific markers, the spatial repartition of prosome within the sarcomeric structure was established. Interestingly, the two major transversal labeled bands observed were found located on both sides of the M band, and the minor one at the level of the Z band. This characteristic cytodistribution seems directly related to the final stage of differentiation of muscle cells**; its exact biological significance remains to be explored.

* Scherrer K and Bey F. Prog.Nucl.Ac.Res.Mol.Biol.(in press).

** Foucquier et al. (Abstracts, present congress).

Mo - 205 STUDY ON LYSOSOMAL DEGRADABILITY OF PURIFIED STORAGE GRANULES FROM ANIMAL MODELS OF BATTEN'S DISEASE (neuronal ceroid lipofuscinosis) BY CULTURED CELLS

M. Elleder

Hlava Institute of Pathology, First School of Medicine, Prague, Czech Republic

Various cell cultures were exposed for 24 hours to a suspension of purified storage granules from ovine ceroid lipofuscinosis and canine ceroid lipofuscinosis, washed repeatedly and incubated for a period up to three weeks. The storage granules were easily phagocytosed by all the cell types cultivated (C6 rat glioma line, rat cardiomyocytes, human fibroblasts). Their internalization was checked by SEM and TEM. Their further fate inside the cultured cells was followed by autofluorescence, phase contrast and by transmission electron microscopy. Intracellular presence of the storage granules never led either to any toxic phenomena in the cultured cells, or to inhibition of their proliferating capacity. Neither of the so far cultured cells was able to degrade the phagocytosed storage granules. This points to their undegradability in the lysosomal system. The resistance may be due to physical barrier caused by hydrophobic nature of the stored subunit c of the mitochondrial ATP synthase.

CHANGES OF MITOCHONDRIAL ATPase DURING LIVER REGENERATION ARE AGE-DEPENDENT

Mo - 206 M. Kalous^a, H. Rauchová^a, Z. Drahota^a, Z. Červinková^b, F. Guerreri^c and S. Papa^c

^aInstitute of Physiology, Academy of Sciences of Czech Republic, Prague, ^bDepartment of Physiology, Faculty of Medicine, Charles University, Hradec Králové, Czech Republic, ^cInstitute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

Loss of hepatic parenchyma produced surgically or by chemical treatment induce the process of regeneration. During several days the original mass of hepatic tissue is restored. The whole process is coupled with dramatic changes of energetic metabolism; at first the return to glycolysis as to the main source of energy is followed by comeback to oxidative phosphorylation.

We have found that activity of mitochondrial ATPase during retrodifferential phase of liver regeneration decreases together with decrease of content of the β subunit of F_1 sector. Redifferentiation phase is characterized by increase of both parameters until they achieve the original value. For K_M of this enzyme we didn't find any significant changes during the whole followed period. Alterations of mitochondrial ATPase during liver regeneration is age-dependent, both retrodifferentiation and redifferentiation are longer in aged rats than in young ones.

T_3 was found to affect liver regeneration, we didn't find decrease in H^+ -ATPase in regenerating liver of T_3 treated animals, characteristic for retrodifferential phase in normal animals. Amount of c subunit of F_1 sector in both control and T_3 treated animals was evaluated immunologically and it seems not to be changed during liver regeneration.

Mo - 207 PROGRESS IN MULTIPARAMETER DIGITAL IMAGING OF RIBOSOMAL RNA GENE FUNCTION

M.F. Trendelenburg^a, O.V. Zatsepin^b, D. Rudolph^c, G. Schmahl^c, and H. Spring^a

^aBiomedical Structure Analysis Group (0195), German Cancer Research Center, D-69120 HEIDELBERG, Germany; ^bA.N. Belozersky Institute of Phys. & Chem. Biology, Moscow State University, MOSCOW 110899, Russia; ^cForschungseinrichtung Röntgenphysik, Universität Göttingen, D-37079 GOTTINGEN, Germany

By contrast to the high amount of information available on sequence and molecular organization of ribosomal DNA transcription in *Xenopus laevis*, much less is known about *in situ* organization of actively transcribing rRNA genes within the intact nucleolus. The fine structural organization of active genes is 'hidden' within very dense masses of surrounding ribonucleoprotein particles (rRNP's) and transcription associated protein complexes.

The advent of advanced digital light microscopy instrumentation, e.g. video-enhanced contrast microscopy and confocal laser scan microscopy has allowed to carry out multiparameter imaging of specific nucleolar compartments in the *in situ* situation.

More recently, a major progress was made in both, instrumentation and specimen preparation techniques for X-ray microscopy. For hydrated biological specimens there are, at present, two optical contrast modes available using the Göttingen X-ray microscope: amplitude contrast as well as phase contrast imaging. Results obtained with these novel types of microscopy will be compared to data obtained from transmission electron microscopy and scanning transmission electron microscopy.

CANCELLED

**EXPRESSION OF PROTEIN TYROSINE KINASE
Mo-209 pp60^{v-src} IN DICTYOSTELIUM DISCOIDEUM
RESULTS IN DEVELOPMENTAL DEFECTS**

F. Púta^a, W. Nellen^b, A. Schweiger^b, L. Krpejšová^a,
A. Blahúšková^a, A. Markoš^a, and P. Folk^a

^a Department of Physiology, Charles University, Viničná 7, Praha 2,
Czech Republic; ^b Max Planck Institute for Biochemistry, Martinsried
bei München, Germany.

The cellular counterpart of the viral protein tyrosine kinase pp60^{v-src} is found in multicellular organisms; its deregulation is oncogenic. Here we express the viral form of the kinase gene in *Dictyostelium discoideum*, a unicellular ameba capable of forming multicellular aggregates.

The gene of v-src from Rous Sarcoma Virus was modified at its 5' end in order to introduce codons preferred in *Dictyostelium*. The gene was then cloned into the pVEII expression vector under the control of discoidin promoter. The use of this promoter allows expression during axenic growth and early development. Transformants were selected on 10 µg/ml Geneticin (G418). Northern and western blot analyses showed the presence of both src-specific mRNA and pp60^{v-src} protein in the cell extracts. No change in protein tyrosine phosphorylation pattern was observed.

Clones producing higher levels of the pp60^{v-src} kinase were then isolated by increasing the G418 concentration. All these clones develop slower as compared to controls and show morphological aberrations (premature stalk formation and defective basal discs). They become arrested in the tight aggregate stage if developed on unbuffered agar. The results document expression and characterization of the pp60^{v-src} kinase in a heterologous system such as *Dictyostelium discoideum* and the effects it has on *Dictyostelium* development.

CANCELLED

**Mo-211 DYNAMIC MOVEMENTS OF THE RNA SUBUNITS
OF RNase P AND MRP RNase VISUALIZED IN
LIVING CELLS.**

T. Pederson, M.R. Jacobson, L.G. Cao and
Y.L. Wang

Cell Biology Group, Worcester Foundation for
Experimental Biology, Shrewsbury, Massachusetts 01545 USA

RNase P is a ribonucleoprotein enzyme that processes transfer RNA precursor molecules. We have introduced the RNA subunit of RNase P (hereafter "H1 RNA") into the nucleus of mammalian cells. Rhodamine-tagged H1 RNA moves very rapidly (within 1-5 minutes) into nucleoli and subsequently is observed to move from nucleoli into the nucleoplasm. The nucleolar sites of H1 RNA are immunoreactive with fibrillarin antibody. U2 and U6 snRNAs and pre-tRNA do not display nucleolar localization, but remain in the nucleoplasm following nuclear microinjection. Nuclear fractionation experiments reveal that the great majority of endogenous H1 RNA is present in the nucleoplasm. Thus, we interpret the rapid initial nucleolar localization of microinjected H1 RNA to reflect a transient phase in RNase P biosynthesis.

We have carried out similar experiments with the RNA subunit of MRP RNase, a ribonucleoprotein enzyme that has been proposed to produce RNA primers for mitochondrial DNA replication. However, the great majority of MRP RNA is found in the nucleolus, where it appears to participate in ribosomal RNA processing. After nuclear microinjection of rhodamine-tagged MRP RNA we find that, like RNase P RNA, it moves very rapidly to nucleoli. Nucleolar localization is dependent on the T antigen binding site in MRP RNA. At later times after nuclear microinjection we also observe MRP RNA appearing in multiple, motile cytoplasmic structures which we regard to be mitochondria. When MRP RNA is injected into the cytoplasm, it enters the mitochondria directly, with no detectable nuclear import.

**Mo-212 DETECTION OF NUCLEIC ACIDS WITHIN POLYTELE
CHROMOSOMES OF SALIVARY GLANDS FROM
CHIRONOMUS TENTANS BY IMMUNOELECTRON
MICROSCOPY**

M. Thiry⁺ and B. Daneholt⁺⁺

⁺Laboratory of Cell and Tissue Biology,

University of Liège, Belgium

⁺⁺Department of Medical Cell Genetics, Medical Nobel
Institute, Karolinska Institutet, S-10401 Stockholm 60,
Sweden

The precise distribution of nucleic acids within polytene chromosomes of salivary glands from *Chironomus tentans* has been investigated, at the ultrastructural level, by immunogold labeling techniques.

Using the *in situ* terminal deoxynucleotidyl transferase-immunogold procedure for detecting DNA, we find evident label over bands and in erbands of chromosomes and over Balbiani rings, and no significant label over the extra-chromosomal nuclear areas and over the cytoplasm.

Furthermore, using anti RNA antibodies and secondary antibodies coupled to colloidal gold, we show that label is deposited not only over the cytoplasm and extrachromosomal nuclear areas but also quite obviously over the Balbiani rings and interbands of chromosomes. No label is seen over bands of chromosomes.

Inside the nucleolus, RNA-positive sites are revealed in both fibrillar and granular parts. In contrast, significant DNA label is exclusively found over the fibrillar zone. Thus, it clearly appears that DNA and RNA are exclusively detected together in well-known chromosomal regions where RNA synthesis takes place in salivary glands: Balbiani rings and interbands. These results suggest that the fibrillar zone of the nucleolus should correspond to site of rDNA transcription.

Mo-213 STRUCTURE AND FUNCTION OF THE RNA PROCESSING MACHINERY OF TRANSCRIPTS OF SPLIT GENES

R. Sperling^a, E. Miriami^a, C. Bessudo-Melamed^b, S. Yitzhaki^b, D. Goldblatt^b, M. Angenitzki^a, and J. Sperling^b

^aDepartment of Genetics The Hebrew University of Jerusalem, Jerusalem Israel; ^bDepartment of Organic Chemistry, The Weizmann Institute of Science, Rehovot Israel

Nuclear transcripts of split genes are processed prior to their export to the cytoplasm while they are packaged in a multicomponent large nuclear ribonucleoprotein (lnRNP) particle. In addition to pre-mRNA, this particle contains components required for splicing, including U snRNPs, hnRNP proteins, and non-snRNP essential splicing factors. Our studies on the biochemical composition and functional characterization of the lnRNP particle indicate that it faithfully represents the nuclear machinery on which pre-mRNA splicing occurs *in vivo*.

The unique and most intriguing feature of the lnRNP particle is our experimental observation that its size and hydrodynamic properties are independent of the size of the RNA it packages. This led us to propose a novel model for the packaging of nuclear RNA polymerase II transcripts in lnRNP particles, whose structural features are determined mainly by their protein component and are thus able to accommodate pre-mRNA molecules of varying sizes. This model is based on biochemical-, functional-, and structural analyses of the lnRNP particles. The proposed model for the packaging of nuclear RNA is conceptually different from that characterizing chromatin, where the beaded structure is dependent on the length of the packaged DNA, and therefore represents a novel type of RNA packaging in the cell nucleus.

CANCELLED

Mo-216 MICROTUBULE AND CHROMATIN CONFIGURATION IN GROWING PIG OOCYTES: EFFECTS OF PHOSPHATASE INHIBITORS

J. Rozinek^a, J. Pet^a, F. Jilek^b

^aRes. Inst. Anim. Prod., ^bUniversity of Agriculture, Praha, Czech Republic

Three distinct chromatin (Cr) configurations and two types of microtubule (MT) arrangement were observed during the growth period in pig oocytes. The oocyte with an internal diameter ranging from 60 to 90 μm exhibited diffuse, fibrous Cr within the nucleus and MT formed a filamentous network with a marked perinuclear array. Cr was more condensed in larger oocytes (diameter 100-110 μm) and MT formed a "sponge-like" structure. This type of MT arrangement is maintained in fully grown oocytes (diameter 120 μm) in which Cr is condensed around the nucleolus in the shape of a ring or a horseshoe.

After meiosis resumption in fully grown oocytes the typical sequence of changes in Cr configuration and MT arrangement was seen. Condensed chromosomes formed a metaphase I plate and MT formed a meiotic spindle. Such oocytes formed numerous cytoplasmic asters in response to the taxol treatment. The induction of meiosis resumption by caliculin A (CAL) (100 Nm) occurred without establishing M-phase MT arrangement. Similarly, okadaic acid (OA) (1 μM) induced meiosis resumption but the M-phase MT arrangement is partially established.

Growing oocytes smaller than 80 μm did not resume meiosis spontaneously and their maturation cannot be induced by treatment with CAL or OA. Despite partial condensation of Cr within the nucleus, their MT arrangement is not influenced after CAL or OA treatment. In larger oocytes meiosis resumption is significantly elevated after CAL or OA treatment but their MT arrangement remained unchanged.

CANCELLED

Mo-217 FOOD RESTRICTION PRESERVES THE MITOCHONDRIAL FUNCTIONALITY IN PROLIFERATING RAT SPLENOCYTES.

C. Pieri, R. Recchioni, F. Moroni, M. Marra and F. Marcheselli

Cytology Center, Gerontological Research Dept. of I.N.R.C.A., Via Birarelli, 8. 60121 Ancona, Italy.

We examined the effect of food restriction on the mitochondria of resting and proliferating rat splenocytes, measuring the membrane potential and mass these organelles, by means of the specific fluorescent probes Rhodamine-123 and Nonyl Acridine Orange respectively. Food restriction was applied on an every other day schedule (EOD) starting from the age 3.5 months. The ad libitum fed (AL) animals were killed when they were 4, 11 and 24 months old, whereas the EOD rats were killed at 11 and 26 months. Resting lymphocytes from AL fed rats showed an age-dependent increase of both membrane potential and mass of their mitochondria. However, the mitochondrial mass increased to a larger extent when compared to the membrane potential, resulting in a decrease of the respiratory quotient (RQ), i.e. of the respiratory activity per unit of mitochondrial mass. In EOD fed animals, the mitochondrial membrane potential was lower and the mitochondrial mass was higher than in the corresponding age-matched controls, resulting in a further decrease of RQ. Following mitogenic stimulation, most of the cells from young and adult AL fed rat showed an increase of membrane potential and mass of their mitochondria. On the contrary about 50% of cells from old AL fed rats had depolarized organelles after 72 hours from the stimulation. Food restriction was able to prevent these alterations allowing the majority of cells, also from old animals, to maintain the hyperpolarization of their mitochondria during the three day culture.

Mo-219 NUCLEAR FACTORS PRE-DETERMINE THE TRANSLATIONAL STATUS OF mRNA IN XENOPUS OOCYTES.

J. Sommerville, M. Ladomery & M. Braddock*
School of Biological and Medical Sciences,
University of St. Andrews, St. Andrews, Fife, KY16 9TS,
Scotland and *Department of Biochemistry, University of
Oxford, Oxford, OX1 3QU, England.

In developing *Xenopus* oocytes, molecules of mRNA are directed either into polysomes or into a non-translating pool of storage particles to be used during oocyte maturation or early embryogenesis. An open question is to what extent the translation programme is pre-determined by the earlier history of protein binding.

Our approach has been to examine the properties of different classes of mRNA-associated proteins (1) and to determine whether particle heterogeneity might arise through differential binding of proteins during transcription/splicing on chromatin loops and during subsequent transport of mRNA to the cytoplasm.

Of the abundant mRNA-associated proteins, two classes have been examined in detail: the heat-stable phosphoproteins pp60 and pp56 which are closely related to the Y-box family of transcription factors (reviewed, 2), and the heat-labile, tyrosine-rich proteins p54 and p52 (3). In addition, the rôle of mRNA-associated protein kinase activity in maintaining the stability of the storage particles has been assessed.

- (1) Marello, K. *et al.* (1992) *Nuc. Acids Res.* **20**, 5593-5600.
 (2) Sommerville, J. (1992) *BioEssays* **14**, 337-339.
 (3) Ladomery, M. *et al.* (1994) In preparation.

Mo-218 CONTROL OF THE AGING OF Vicia faba SEEDS BY MEANS OF STORAGE EFFECT.

K. Mičieta, G. Murin

Institute of Cell Biology, Comenius University,
Révová 39, 811 02 Bratislava, Slovakia

Plant seeds are only known, unique living system which aging we can regulate without using artificial drugs or other agents. Simple impact of water content of seeds on their activity is sufficient tool for speeding or slowing down the aging process. We know that first hours of germination plays a crucial role in manifestation of chromosomal damage of the old seeds. By means of the experimental storage of seeds at reduced water content it could be prolonged from hours to days. This period is (or is not) favourable for pre-replicative DNA repair according to the level of reduced water content (50% vs. 25-30%). Thus after 7-days of storage we can found 3-4 times lower frequency of chromosomal aberrations and higher viability of seeds (50%) or vice versa (25-30%). This effect was studied in five different annual sets of old broad bean seeds.

Mo-220 A FLUORESCENT EXPRESS-ANALYSIS FOR EVALUATION OF PHYSIOLOGICAL INTEGRITY OF CELLS

E. Melnikova, A. Kazantsev
Institute of Cell Biophysics RAS,
Pushchino, Moscow Region, Russia

A fluorescent express method for evaluation of the physiological integrity of cells is suggested. The method was tested on 1) sperm cells after cryoconservation, 2) blood lymphocytes after γ -irradiation, 3) plant cells after ecological stress.

The method is based on computer analysis of the differences in fluorescence spectrum parameters of individual cells or cell populations. A double canal microfluorometer Radical DMF-2 interfaced to PC/AT is used to measure fluorescence intensities from different samples in two separate wave length bands. The instrument is based on fluorescent microscope with a double canal fluorescence sensor assembly. Measuring and data processing are managed by the software system interacting with the researcher, which is operating at both the microscope and the computer. The statistical information about the population under investigation, the scattergram on the coordinate plane of the two fluorescence intensities (x,y) and the corresponding histograms of distributions of x, y and y/x ratio are displayed immediately in process of measurement. The data arrays are filed by the computer.

**STRUCTURE FUNCTION RELATIONSHIP
OF THE PERIPHERAL AND NEURONAL
MO - 221 CANNABINOID RECEPTORS**

Bayewitch M.¹, Levy R.¹, Barg J.¹, Matus-Leibovitch N.¹, Mechoulam R.², and Vogel Z.¹ Dept. of Neurobiology, Weizmann Institute of Science, Rehovot¹ and Dept. of Natural Products, Faculty of Medicine, The Hebrew University of Jerusalem, Israel.²

Two cannabinoid receptors, neuronal and peripheral, have recently been identified. The two receptors share 44% amino acid identity and the homology increases to 68% within the transmembrane domains (Monro, et al. *Nature* 365:61, 1993). We have transiently expressed the human peripheral and rat neuronal cannabinoid receptors in COS 7 cells and assessed the binding of various cannabinoid ligands. We found that the high affinity ligand for the brain cannabinoid receptor, ³H-HU243, binds to the neuronal and peripheral receptors with K_d values of 40.3 and 81.8 pM, respectively. In addition, heterologous competition binding assays with the brain endogenous cannabinoid ligand, anandamide, shows slight preferential binding to the neuronal over the peripheral receptor, with K_i values of 252 ± 47 and 581 ± 111 nM, respectively. Another endogenous cannabinoid ligand, 2-arachidonyl glycerol was recently identified in dog small intestines (Mechoulam et al., unpublished). We have found that this compound binds to both neuronal and peripheral cannabinoid receptors with K_i values of 610 ± 75 and 1400 ± 172 nM, respectively. Several cannabinoid ligands exhibit differential binding affinity to the two receptors. Among these is cannabinol with K_i values of 1336 ± 145 and 211 ± 26 nM. Both anandamide and 2-arachidonyl glycerol inhibited adenylyl cyclase in N18TG2 neuroblastoma cells and in CHO cells transfected with the neuronal receptor. The signal transduction of the peripheral receptor is under investigation. This work was supported by grants from the Anti-drug Authority of Israel, and from the National Institute of Drug Abuse USA.

**COMPUTER ANALYSIS OF SEVERAL MEMBERS
OF GRS REPETITIVE SEQUENCE FAMILY OF
TOBACCO REVEALS A CONSERVED DNA
CURVATURE PATTERN**

Mo - 222 R. Královics, B. Brzobohatý and M. Bezdečk

Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic

Several members of the GRS repetitive sequence family have been cloned and sequenced. Their anomalous gel mobility in polyacrylamide gels gave an evidence that these sequences are curved. The nearest-neighbour (or wedge) model of DNA curvature [Bolshoy, A. et al. (1991), *Proc. Natl. Acad. Sci. USA* 88, 2312-2316] has been applied to predict the curvature patterns of individual GRS family members with known nucleotide sequence. The results suggest a unique curvature pattern for all analysed sequences. A dominant curve is predicted at the 5' end of the sequences where oligo(dT) and oligo(dA) sequence motifs are found in phase with helical turn (10-11 bp). These sequence motifs are known to be the major determinants of intrinsic DNA curvature. A minor curve is also predicted at the center of the GRS sequences. Sequences responsible for this helix axis deformation are of the type oligo(dA,dT) out of the helical turn with a d(A_nT_m) sequence motif. Higher order structures are also predicted for tandemly arranged repeats of GRS units. Possible information content of this structure is discussed with respect of nucleosome and overall chromatin structure.

**CHARACTERIZATION, SUBCELLULAR
LOCALIZATION AND POTENTIAL ROLES FOR
GI2/GI3 IN PREADIPOCYTES FROM RAT
MO - 223 ADIPOSE STROMA VASCULAR FRACTION**

D. Denis, P. de Mazancourt and Y. Giudicelli
Dept. of Biochemistry, Faculty of Medicine
Paris-Ouest, C.H.I., 78303 Poissy, France

Proliferation of some fibroblast cell lines such as the NIH-3T3 has been shown to be dependent on some G proteins. This has led us to study G proteins in rat preadipocytes in order to determine whether these proteins could also play a role in preadipocyte proliferation. If so, G proteins could be potentially involved in the physio-pathological mechanisms underlying hyperplastic obesity.

1 - Immunoblotting experiments have revealed the presence of Gi2 and Gi3 but not Go and G1 in the 100,000 g pellet prepared from confluent preadipocyte homogenates.

2 - Indirect immunofluorescence was used to investigate the subcellular distribution of Gi2 which was found to be mainly associated with perinuclear sites.

3 - Exposure of cultured preadipocytes to Bordetella Pertussis toxin (which functionally inactivates Gi2 and Gi3) reduced by 30 % the cellular growth stimulated by 8 % FC serum.

These results lead to conclude that Gi2 and/or Gi3 transduce(s) some of the mitogenic signals present in serum. Moreover, the peculiar subcellular localization of Gi2 and Gi3 in preadipocytes suggests that some of the Gi2 and Gi3 functions could be related to their association with subcellular organelles other than the plasma membrane.

**DIFFERENTIAL INHIBITION OF APICAL
TRANSPORT PROCESSES IN LIVER BY CSA AND ITS
NON-IMMUNOSUPPRESSIVE ANALOG SDZ PSC 833.**

Mo - 224 H. Swatonek, J. Graf, L. Gajzik, K. Tanczos and T. Thalhammer, Dept. General and Exptl. Pathology, Vienna University, Vienna, Austria.

P-glycoprotein (P-gp), which contributes to multidrug resistance (MDR) in tumor cells, operates as a membrane ATPase which extrudes various lipophilic compounds and, in addition, it mediates Cl⁻-dependent cell volume regulatory decrease (VRD).

In the isolated perfused rat liver, we studied the effects of the P-gp inhibitors cyclosporin A (CsA) and the non-immunosuppressive analogue SDZ PSC 833 on the biliary excretion of the fluorescent P-gp substrates rhodamine-123 (rho) and doxorubicin (dox) and their influence on VRD. The inhibitory effect of PSC on the excretion of rho and dox was 100 times more pronounced than that of CsA. Furthermore, PSC had no effect on the excretion of i) taurocholic acid, ii) bilirubin and iii) horseradish peroxidase (HRP), a marker for hepatic fluid phase endocytosis. However, both cyclosporins inhibited the excretion of sulfobromophthalein-glutathione. Moreover, PSC hardly binds to cyclophilin, a protein which mediates the immunosuppressive activity of CsA, however both CsA and PSC inhibited photoaffinity-labelling of immunoprecipitated liver P-gp with azidopine, indicating their binding to P-gp. In addition, PSC and CsA as well as a series of P-gp substrates including rho and dox inhibited isolated liver cell VRD following cell swelling in hypotonic media.

Our data suggest that P-gp, which mediates biliary excretion of lipophilic compounds and contributes to liver cell volume regulation, is more effectively inhibited by PSC than by CsA. At inhibitory doses, PSC did not effect bile flow; bile salt, bilirubin and HRP excretion. Our data indicate, that the inhibitory effect of PSC is restricted to a few transport systems.

The different effects of PSC and CsA on hepatic transport systems could be of relevance for clinical application.

**YEAST CELLS IN POPULATION HAVE
NONUNIFORM CYTOPLASMIC pH VALUES**

Mo - 225 P. Cimprich, J. Slavík and A. Kotyk

Institute of Physiology, CzAcadSci, Vídeňská 1083,
CZ-142 20 Prague 4, Czech Republic

Cytoplasmic pH values of individual cells of *Saccharomyces cerevisiae* seem to fit a Gaussian distribution with a mean value of pH 6.0 and a halfwidth of 0.5 pH unit in the case of culture in stationary phase, as revealed by ratio imaging microscopy using carboxyfluorescein and (bis-carboxyethyl)carboxyfluorescein fluorescent pH probes. In freshly harvested cells there are indications of the coexistence of two cell subpopulations differing in both the mean cytoplasmic pH value and the intensity of fluorescence staining by carboxyfluorescein. Intracellular distribution of pH of individual cells seems to be nearly homogeneous, in some cases with the exception of large organelles. The viability of the observed cells has been confirmed by vital staining with methylene blue.

B-MYB FUNCTION IN SURVIVAL AND DIFFERENTIATIVE POTENTIAL OF NEUROBLASTOMA CELLS

Mo - 226

G. Raschellà¹, A. Negroni¹, A. Sala², S. Pucci¹, A. Romeo¹ and B. Calabretta²

¹ Department of Environmental Sciences, Radiobiology Unit, ENEA CRE Casaccia, 00060 Rome, Italy. ² Jefferson Cancer Institute, Philadelphia, PA 19107, U.S.A.

B-myb gene belongs to a family of transcription factors which also includes A-myb and c-myb. B-myb is highly expressed in human neuroblastoma cells. We demonstrate that B-myb expression is down-regulated during neural and glial differentiation pathways induced by retinoic acid (RA) *in vitro*. This modulation is an early event, taking place after six hours from the beginning of RA treatment and is maintained also at late times of induction (10 days). Run-on experiments indicate that B-myb regulation occurs, at least in part, at transcriptional level. We have transfected neuroblastoma cell line LAN-5 with an expression vector in which a 714 bp fragment from the human B-myb cDNA was cloned in antisense orientation. Expression of B-myb antisense transcript does not allow the rescue of viable cells suggesting a very important role of B-myb for the survival of neuroblastoma cells. Furthermore, a full-length B-myb cDNA was cloned in the polylinker region of an expression vector downstream the cytomegalovirus promoter. This construct was used to stably transfect a neuroblastoma cell line. The analysis of B-myb transfected clones demonstrate that the constitutive expression of B-myb inhibits neural differentiation induced by RA treatment. In fact, RA-treated B-myb transfectants, unlike the parental cell line and controls, do not show any substantial change in morphological and biochemical markers of neural differentiation. These results indicate that B-myb plays a functional role in the differentiative potential of neuroblastoma cells, raising the possibility that is one of the nuclear targets in the cascade of events leading to cellular differentiation.

**HIGHER LEVEL OF C-MYC mRNA IN
REGENERATING MOUSE TESTES AFTER
LOCAL X-IRRADIATION DETERMINED BY
COMPETITIVE RT-PCR**
R. Amendola

Mo - 227
Department of Environmental Sciences, Radiobiology Unit, Enea CRE-Casaccia, 00060 Roma-Italy

Expression of the *c-myc* proto-oncogene has been well related with transition of quiescent cells (G₀ phase) to proliferate through G₁ phase of cell cycle, though *in-vitro* experiments have suggested that cells committed to ultimate a differentiation process with a few mitotic divisions do not need *c-myc* expression. In murine spermatogenesis the level of *c-myc* transcripts does not correlate with the rate of cellular division. Higher amount of *c-myc* mRNA has been evaluated at 3 days post-partum, then it decrease to disappear at 30 days p.p., when spermatogonia A and B are proliferating. In order to determine if the expression of *c-myc* may be activated in proliferation out of terminal differentiation, a local, single 5 Gy X-ray dose has been delivered to induce regeneration (mitotic divisions of supposed staminal spermatogonia, As, to reproduce themselves) and repopulation (mitotic divisions to differentiate in aplid population) in 90 days old C57Bl mouse testes. Damage and restoration of spermatogenesis has been followed by relative body/testis weight determination, and histological examination. *c-myc* expression has been quantitated by competitive RT-PCR. Proliferative status has been determined by histon-H3 Northing Blot analysis. *c-myc* mRNA level is ten fold higher at 3 days after irradiation in treated animals than control (5×10^3 copies vs. 5×10^2). An increasing number of copies is expressed until 10 days (2×10^4), but they promptly decrease to the basal level of 5×10^3 copies for irradiated mice since 13 days to 60 days. Interestingly, the expression of H3 detects S-phase only in testes at 60 days from damage. *c-myc* plays a key-role to drive proliferation/differentiation processes, and innovative technique as competitive RT-PCR, is a powerful tool to study transcription of low copies genes.

**GABA-INDUCED CHLORIDE CURRENTS
IN ANTERIOR PITUITARY CELLS**

Mo - 228
H.Zemkova, J.Krusek and J.Vanecek
Institute of Physiology, Academy of Sciences
of the Czech Republic, Praha, Czech Republic

The release of hormones from endocrine cells of anterior pituitary gland is controlled by peptides originating from the hypothalamus, but hormone secretion is regulated at the pituitary level also by γ -aminobutyric acid (GABA). Numerous morphological, histochemical and biochemical studies have established the presence of a nonclassical GABA(A) receptor in anterior pituitary cells but an electrophysiological study is still lacking. In the present report we studied the effect of GABA on anterior pituitary cells in culture using the patch-clamp technique in a whole cell configuration. With internal Cs and at holding potential of -60 mV, GABA elicited an inward current in about 80 % of anterior pituitary cells. The GABA dose-response curve showed that the GABA EC₅₀ was 22.9 ± 4.2 μ M and the Hill number was 1.8 ± 0.2 . The current responses to GABA (10 μ M) were almost linearly related to the membrane potential in the range between -80 mV and +60 mV and were inhibited by micromolar concentrations of bicuculline, picrotoxin and zinc. Diazepam increased the GABA responses to 220 % with EC₅₀ of 1.0 ± 0.4 μ M and pentobarbital to 195 % with EC₅₀ of 14.4 ± 8.2 μ M. Pentobarbital (100 μ M) itself did not induce any inward current. We conclude that the nonneuronal GABA(A) receptor in anterior pituitary does not differ in its sensitivity to inhibitors and potentiating drugs from the GABA(A) receptors in CNS neurones.

Mo-229 STRUCTURE AND FUNCTION OF THE F_0 COMPLEX OF THE ATP SYNTHASE (F_1F_0) OF *ESCHERICHIA COLI*
K. Altendorf and G. Deckers-Hebestreit

Universität Osnabrück, Fachbereich Biologie/Chemie, D-49069
Osnabrück, Germany

The ATP synthase of *Escherichia coli* consists of a peripheral F_1 complex, which carries catalytic centers (five subunits: α , β , γ , δ and ϵ) and a membrane-integrated, proton-translocating F_0 complex (three subunits: a , b and c). The stoichiometry of the different subunits in the enzyme complex has been determined to be $a,b_2c_{10\pm},\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. Analyses of mutant strains supported the notion that conserved amino acid residues in subunit c (Asp61) and subunit a (Arg210, Glu219, His245) may be directly involved in H^+ translocation. This observation calls for an intimate interaction between both subunits, which has been demonstrated by labelling of subunit c and the ac complex, both reconstituted into liposomes, with the hydrophobic, photoactivatable reagent 3-(trifluoromethyl)-3-(m-[¹²⁵I]iodophenyl diazirine. Analysis of the labelling pattern of subunit c in both cases revealed a dramatic reduction in the extent of labelling when going from subunit c to the ac complex. Obviously, subunit a has the capacity to organize an oligomeric arrangement of subunit c. Subunits a and b receive also label, which lends support to the notion that the a,b_2 moiety is located outside the subunit c oligomer, although the labelling pattern of subunit a has so far not been determined. This view that the structure of F_0 is of asymmetric nature received further support from recent studies, in which detergent-solubilized F_0 and ac complexes have been investigated by transmission electron microscopy applying the electron spectroscopic imaging (ESI) mode.

Mo-230 PHOSPHORYLATION OF MAP1B IN GROWING AXONS
Philip R. Gordon-Weeks
Developmental Biology Research Centre,
King's College London, U.K.

Axonal growth and growth cone function depend critically on an appropriately organized array of microtubules. Recent experiments indicate a crucial role for the microtubule-associated protein (MAP) 1B in these events. We have identified and characterised a novel phosphorylation epitope on MAP1B that is only expressed in growing axons in vertebrates (1, 2). MAP1B is phosphorylated at multiple sites during development and the site that we have identified is one of the earliest to be expressed. Using a recombinant MAP1B protein we have established an in vitro assay for the kinase responsible for phosphorylating this novel site on MAP1B. Our assay has enabled us to identify the phosphorylation site and to characterise the kinase.

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Tu-1 SPONTANEOUS DIFFERENTIATION OF
MOUSE EMBRYONIC STEM CELLS

V. Horák^a, J.E. Fléchon^b and A. Moens^c

^aInstitute of Animal Physiology and Genetics,
277 21 Liběchov, Czech Republic; INRA, ^bBiologie Cellulaire,
^cBiologie du Développement, 78350 Jouy-en-Josas, France

Mouse D3 embryonic stem (ES) cells were cultivated in medium (Robertson 1987) with LIF at 2000 i.u./ml. Spontaneous differentiation was induced by omitting LIF. Indirect immunofluorescence was used to localize various cytoskeletal proteins. Alkaline phosphatase (AP) was demonstrated histochemically (Lojda et al. 1976).

Undifferentiated ES cells showed AP activity and a dense α -tubulin network in whole cytoplasm. F-actin and smooth muscle myosin demonstrated similar cell distribution. Antibodies against intermediate filament proteins (IFPs) gave no reaction.

Spontaneous differentiation of ES cells started already after 1 day of cultivation without LIF. ES cells gradually lost their round shape, increased rapidly in size, migrated from cell clusters to the surroundings and changed their cytoskeletal protein profile. Two main cell types (fibroblast- and endoderm-like cells) were distinguished using vimentin and cytokeratin 18 (IFPs) expression and F-actin distribution. Several minor cell types (endothelial and neural cells, myoblasts) were occasionally observed. Some colonies of ES cells still retained AP activity at the 9th day of cultivation without LIF.

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Tu-3 DETERMINATION OF THE MICROTUBULE NUCLEATION CENTRES SIZE WITH TARGET THEORY

Sorochinsky B.V. and Prokhnevsky A.I. Department of Biophysics and Radiobiology, Institute of Cell Biology and Genetic Engineering, Ukrainian Academy of Sciences, 148 Zabolotnogo st., Kiev, 252143, Ukraine.

Isolated microtubules were irradiated before polymerization and dose-dependence of polymerization processes was investigated. Then D_{37} value, which need for the calculation of the target size has been determined. The curves were built using the absorption level for 5th minute after initiation of polymerization because the aim was to determine the intermediate structures' size. The formula used for the calculation was followed: $MW = 5.8 \times 10^{11} / D_{37}$ Daltons. After the substitution of the D_{37} value the molecular weight of nucleation centres was obtained. Their sizes correspond to 69 (irradiation at 0 °C) or 77 (irradiation at 20 °C) tubulin dimers. Thus Target theory allows to obtain the real size for intermediate structures appeared during first steps of microtubule polymerization.

Tu-2 CYTOCHALASIN B BINDING TO THE BARBED END OF ACTIN FILAMENTS IS NOT A PREREQUISITE FOR FILAMENT SHORTENING

C. Stournaras^a, A. Tsapara^a, A. Gravanis^b, A.N. Margioris^c, and P.A. Theodoropoulos^a

^aDepartments of Biochemistry^a, Pharmacology^b and Clinical Chemistry^c, School of Medicine, University of Crete, GR- 71110 Heraklion, Greece

It is generally accepted that cytochalasin B (CB), as well as other cytochalasins, shorten actin filaments by blocking monomer addition at the fast-growing ("barbed") end of these polymers. Despite the predominance of this mechanism, recent evidence suggest that other interactions may also occur between CB and F-actin. To further investigate this possibility we have employed an actin derivative, prepared by substitution at Cys³⁷⁴ by a glutathionyl residue. We demonstrate here that CB did not significantly bind to glutathionyl F-actin under several ionic conditions. We further show that in the presence of CB the glutathionyl-F-actin exhibits a significantly higher ATPase activity than the non modified F-actin. These data argue that the incorporation of glutathionyl groups prevents the high-affinity binding of CB to the barbed end of actin filaments. This is probably due to a decreased hydrophobicity of the CB binding site by the introduction of the hydrophylic glutathionyl residue, as shown by the partial recovery of the binding capacity of CB to glutathionyl-actin filaments in the presence of glycerol. Despite the lack of substantial binding at equilibrium, we have found that the addition of CB to glutathionyl-F-actin results in extensive fragmentation of the filaments, as demonstrated by electron microscopy and by a significant reduction of the relative viscosity of actin solutions. These results are consistent with the idea that CB shortens glutathionyl-actin filaments by a mechanism distinct from barbed end capping.

Tu-4 IN G₀-ARRESTED CELLS EUKARYOTIC ELONGATION FACTOR 2 REMAINS TO CO-Localize WITH INTERMEDIATE FILAMENTS AFTER MICROTUBULE DISASSEMBLY

E.A.Shestakova and I.P.Gavrilova

Institute of Protein Research, Academy of Sciences of Russia, 142292, Pushchino, Moscow Region, Russia

Indirect immunofluorescent microscopy studies of intracellular distribution of eukaryotic elongation factor 2 (eEF-2) and ribosomes have shown co-localization of these components of protein-synthesizing machinery with actin microfilament bundles in fixed cycling mouse embryo and human skin diploid fibroblasts (Gavrilova et al., 1987, Cell Biol. Int. Rep., 11, 745-753; Shestakova et al., 1991, Cell Biol. Int. Rep., 15, 75-84; Shestakova et al., 1993, Cell Biol. Int., 17, 409-416). In G₀-arrested fixed human skin diploid fibroblasts eEF-2 and ribosomes were distributed mainly along the intermediate filaments and/or microtubules (Shestakova et al., 1993, Cell Biol. Int., 17, 417-424). We found that after microtubule disassembly with 10 μ g/ml nocodazole eEF-2 remained to be co-localized with intermediate filaments in G₀-arrested cells. It is likely that intermediate filaments can be involved in intracellular organization of eEF-2 and thus protein-synthesizing machinery in G₀-arrested fibroblasts.

Tu-5 THE REGULATION OF MICROTUBULES DURING OOCYTES IN INSECT TELOTROPHIC OVARIOLES
J.D. Lane and H. Stebbings

University of Exeter, Department of Biological Sciences, Washington Singer Laboratories, Perry Road, Exeter, EX4 4QG, U.K.

During the previtellogenetic stages of oogenesis in the telotrophic ovaries of hemipteran insects, the oocytes, which are arrested at the first meiotic prophase, are supplied by nurse cells via elongated cytoplasmic processes known as nutritive tubes. The latter contain many thousands of parallel microtubules along and between which the nurse cell contributions pass, and these may be continuous with the microtubules of the oocyte cortex. At around the onset of vitellogenesis, translocation along the microtubules ceases, the nutritive tube becomes redundant, and there is a change in microtubule dynamics. We have observed that the nutritive tube microtubules first become more closely packed, and this is followed by their depolymerisation. These changes, however, are not temporally linked and appear to be regulated independently. Interestingly, the change in microtubule dynamics within a nutritive tube occurs at about the time that the network of microtubules within the oocyte cortex is also restructured [McPherson, S.M.G. & Huebner, E. (1993) *Tissue Cell* 25: 399-421].

Previous studies of the ovaries of hemipteran insects have not addressed the regulatory signals effecting the alterations in microtubule properties, merely noting that they occur around, or sometime after, the onset of vitellogenesis. Here we have investigated the possible correlation between the restructuring and breakdown of nutritive tube and oocyte cortical microtubules and the assembly of the first meiotic spindle during the progression of the oocyte to arrest at metaphase I.

Tu-7 CULTURE SUBSTRATE INFLUENCE ON KERATINOCYTE DIFFERENTIATION AND CYTOSKELETAL ACTIN DISTRIBUTION

V.V. Gorelik, M.I. Blinova, G.P. Pinaev,

Institute of Cytology, Sci. Acad. Russia, Tikhoretsky av 4, 194064 StPetersburg, Russia

Two-days old newborn rat keratinocytes were plated either in serum-free low-Ca medium or in the complete medium to measure cell differentiation. Two classes of cells were determined with different actin pattern: class 1 with well developed actin bundles and lamella, and class 2 with condensed actin ring around cell margins. The relative amount of cells of the two classes was different on fibronectin and collagen type 1 as compared with collagen type 4 and glass. The actin distribution corresponded to keratinocyte colonies morphology. While colonies on fibronectin, glass and collagen type 4 were condensed, they were loose on collagen type 1.

In the stripping model the amount of basal-like cells remained after epidermal strip removal was revealed on different substrates. All substrates made of the extracellular matrix proteins, namely collagen type 1, fibronectin and EHS-matrigel were found to retain about 30% to 50% of cells in comparison with the control glass substrate. This suggests that the differentiation is accelerated the cells being in contact with extracellular matrix.

Tu-6 CO-EXPRESSION OF GFAP AND VIMENTIN IN ADULT HUMAN BRAIN TISSUE

A. Perželová, I. Máčiková and P. Mráz
Department of Anatomy, Comenius University, Bratislava, Slovakia.

GFAP is a specific component of the astroglial intermediate filaments. Vimentin is a nonspecific intermediate filament protein originally considered to be specific for mesenchyme-derived cells.

The purpose of this study was to report some findings concerning the expression of GFAP and vimentin in adult human cortical gray and white matter. Frozen sections were prepared from ten normal human brain biopsies and were studied by double immunofluorescence. The positively stained cells for GFAP were present in the white matter and in the subpial area. Staining for vimentin was observed only in GFAP-positive cells and in the cells of brain vessels. In glial cell of the cortical gray matter except the subpial area neither vimentin nor GFAP were detected. The intensity of staining was similar for both intermediate filaments.

The immunofluorescence staining of brain tissue shows that astrocytes may contain either both or no intermediate filaments. These results indicate that the significance of detecting GFAP and vimentin as cell type-specific markers may be of limited value.

Tu-8 CO-EXPRESSION OF GFAP AND VIMENTIN IN PRIMARY CULTURES DERIVED FROM ADULT HUMAN BRAIN

I. Máčiková, A. Perželová and P. Mráz
Department of Anatomy, Comenius University, Bratislava, Slovakia.

In previous studies we have shown that GFAP and vimentin were not present in all astroglial cells but only in those located in the subpial area and in the white matter.

In this study we have focused our attention to primary cultures derived from five apparently normal adult human brain biopsies from cortical gray and white matter. The cells were investigated by double immunofluorescence using antibodies against GFAP and vimentin. GFAP-positive cells were found in all cultures from white matter. At confluence these cells reached 0.1% of total cells number. The majority of GFAP-positive cells showed similar morphologic features as fibrous astrocytes; only a small number of cells were of plasmatic or bipolar shape. GFAP-positive cells appeared extremely rare in cultures derived from cortical gray matter. All cells in all cultures were positively stained for vimentin.

Our results show that primary adult human brain cultures contained almost entirely GFAP-/vimentin+ cells. The histogenetic origin of these "glia-like" cells is until now unclear.

Tu-9 INVOLVEMENT OF POLYAMINES BIOSYNTHESIS IN CYTOKINESIS INDUCTION

C.O.Audit^a and C.Aimar^b

^aLaboratoire de Biologie animale (CNRS 15393)
^bLaboratoire d'Immunologie comparée (CNRS URA 1135)
 Université Pierre et Marie Curie - 75252 Paris cedex 05

In dividing animal cells, the cleavage furrow expanding during cytokinesis is generated by a transitory organelle, the actin-containing contractile ring. This ring could be formed by the polymerizing action of polyamines on actin since we have shown that polyamines can induce furrowing as well as bundling of actin in egg cortex.

In this study, the role of polyamine biosynthesis in cleavage induction and early embryonic development has been investigated in Pleurodetes waltl. (amphibian).

During the first division cycle of activated egg, a three-time increased amount of the two main polyamines, putrescine and spermidine, was observed just before cytokinesis. Moreover, the time of first cleavage was dependent on the intracellular polyamine concentration. Injections of polyamines biosynthesis inhibitors caused a delay in egg cleavage and this delay was reversed by injecting polyamines with inhibitors.

Then, the role of polyamine biosynthesis in early embryonic development has been studied. Injections of polyamine inhibitors into fertilized eggs caused abnormal (25%) or delayed (35%) first cleavage. The absence or delay of next cleavages prevented normal development up to the blastula stage.

These results obtained in amphibian eggs, and other results from eggs of different species or animal cells indicate that the induction of cytokinesis is directly linked to the polyamine biosynthesis.

Tu-11 MONOCLONAL ANTIBODY M3 USED IN TPS ASSAY FOR THE QUANTIFICATION OF TPA RECOGNIZES KERATIN 18

J.M.G. Bonfrer¹, E. Groeneveld¹, C.M. Korse¹, A. van Dalen², L. Oomen¹ and D. Ivanyi¹

¹ The Netherlands Cancer Institute/AvL, Plesmanlaan 121, 1066 CX AMSTERDAM, The Netherlands; ² Department of Nuclear Medicine, Groene Hart Hospital, Bleulandweg 10, 2803 HH GOUDA, The Netherlands

TPA (tissue polypeptide antigen), used as a tumor marker in clinical diagnoses and follow-up, was shown to be a degradation product of cytokeratins (CK) 8, 18 and 19. Recently, a new "specific TPA" test was introduced and designated TPS; it is based on the monoclonal antibody (MAb) anti-TPS, M3. We have tested the specificity of this antibody by immunocyto- and immunohistochemistry, gel electrophoresis and immunoblotting.

MAb M3 bound to intermediate filaments of epithelial cells and on tissue sections of various human tissues revealed a staining pattern identical to CK18 specific MAb (DE-K18). On immunoblots of proteins extracted from various epithelial cell-lines, M3 reacted with a 45kD protein corresponding to CK18, and on immunoblots of proteins isolated from MCF-7 culture fluid M3 stained three bands, 45kD, 33kD and 29kD. The same bands were stained with CK18 specific MAb, indicating that they represent CK18 and its degradation products. In contrast to TPA, MAb M3 did not stain CKs 8 and 19, present on immunoblots.

Tu-10 EXPRESSION OF CYTOKERATIN 10, 13 AND INVOLUCRIN AS PROGNOSTIC FACTORS IN LOW-STAGE SQUAMOUS CELL CARCINOMA OF THE UTERINE CERVIX

PFJ van Bommel¹, P Kenemans^{2,3}, TJM Helmerhorst^{2,3}, MPW Gallee⁴ and D Ivanyi¹

¹Department of Obstetrics and Gynaecology, Ignatius Hospital, Breda and ²Free University Hospital, Amsterdam. ³ Department of Gynaecological Oncology, ⁴ Pathology, ⁵ Tumor Biology, The NKI/AVL, Amsterdam.

In low-stage cancer of the uterine cervix several differentiation related markers are variably present and may relate to differences in the behavior of the tumor. An indirect immunoperoxidase technique was applied to formalin-fixed paraffin-embedded tissue sections of 80 FIGO stage IIA primary squamous cell cervical carcinomas for detection of expression of cytokeratin 10 and 13, and involucrin. Comparisons were made on the expression of each of these markers between 40 node-positive and 40 age-matched node-negative patients. Differences in frequency of lesions with expression were also analyzed in relation to histopathological characteristics, recurrence and survival. Expression of cytokeratin 13 and involucrin was associated with tumor-grade ($p=0.01$). We did not find any relation between expression of the markers used and recurrence or survival in the entire group. Within the node-positive group, however, the survival rate of patients with tumors with cytokeratin 13 expression was significantly higher than that of patients with tumors lacking cytokeratin 13 expression ($p=0.02$). Conclusions In low-stage squamous cell cervical cancer cytokeratin 13 expression appears to be of prognostic significance in node-positive patients.

BEHAVIOR OF SPECTRIN IN RED CELLS OF YOUNG AND OLD INDIVIDUALS

Tu-12

N.S. Kosower, Y. Cohen, T. Glaser and Y. Zipser

Department of Human Genetics, Sackler School of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel.

Spectrin is important for the stability and deformability of the red cell membrane. Spectrin was studied in young individuals (20-30 years old) and in old individuals (>70 years of age) with respect to: 1) Ratio of tetramers/dimers; 2) Phosphorylation; 3) Degradation by the protease calpain.

1) Spectrin α & β chains heterodimers (SpT) and oligomers (SpO). Spectrin is extracted from the normal red cell membrane mostly as SpT and SpO (at 4°C), or as SpD (at 37°C). At 4°C, the ratio of SpT/ SpD was 1.5-3/1 in the young and 0.6-1/1 in the old. Spectrin extracted at 37°C showed mostly SpD in both young and old individuals. 2) Spectrin β chain (serine & threonine residues) was phosphorylated to a lesser degree in membranes of old than in those of young individuals. 3) Limited proteolysis by calpain (Ca^{2+} -activated protease) is believed to regulate the behavior of membrane proteins. Spectrin extracted at 4°C from red cell membranes of old people was degraded by calpain more easily than spectrin of young ones. Spectrin extracted at 37°C was degraded by calpain more easily than spectrin extracted at 4°C; at 37°C it was degraded to the same extent in young and old. It appears that the enhanced sensitivity of the spectrin of old individuals to calpain arises from the presence of a higher dimer/ tetramer ratio in spectrin of old people.

The increase in spectrin dimers, diminished phosphorylation and enhanced degradation by calpain indicate age-associated alterations in the protein and/or associated membrane components.

Supported by a grant from the Fritz Thyssen Stiftung

Tu-13 Distinct behaviour of two exocrine glands towards protein secretion in response to microtubule network disturbance

P. ROBIN, M.-N. RAYMOND, and B. ROSSIGNOL

Laboratoire de Biochimie des Transports Cellulaires, CNRS URA 1116,
Université de Paris XI, 91 405 ORSAY Cedex, FRANCE

The role of microtubules in the exocrine secretory process is not yet well established. Contradictory effects of anti-microtubule drugs on intracellular transit and protein secretion are reported (see Ref.). In this work we used microscopic techniques and pulse-chase experiments to compare the involvement of microtubules in the regulated secretory process of two exocrine glands from rat: parotid and exorbital lacrimal glands. In our experiments microtubules were either disrupted by colchicine or nocodazole, or stabilized by taxol. We show that in both cell types, microtubule network is disrupted by 10 μ M colchicine or nocodazole. Nevertheless the effect of these drugs on the release of newly synthesized proteins is radically different in the two tissues; in parotid glands they only weakly delay protein release, triggered by stimulation of either muscarinic or β -adrenergic receptors, but in lacrimal glands, they strongly inhibit protein release. On the other hand we show that in parotid glands 10 μ M taxol has no effect on protein secretion whereas it reduces it in lacrimal glands. This "non-effect" or effect of the drug on the secretory pathway is independent of the nature of the secretagogue used to trigger exocytosis. We conclude from this study that microtubule network integrity is essential for protein secretion in lacrimal glands but not in parotid glands. This result implies that for the same physiological function, i.e. protein secretion, different mechanisms may be involved.

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Tu-15 CYTOSKELETAL PERTURBATION INDUCED BY PARAQUAT

G. Cappelletti, C. Incani, U. Fascio and R. Maci.

Dept. of Biology, University of Milan, Milan, Italy.

Interference with cytoskeletal proteins represents an important event associated with the manifestation of cellular toxicity (Marinovich M., Pharmacol. Res., 1991). Actin cytoskeleton perturbation has been described in cultured cells after treatment with the herbicide paraquat (Cappelletti G. and Maci R., Bull. Environ. Contam. Toxicol., 1993).

In order to better understand the role of actin in the transduction of toxic effect of the pulmonary-specific herbicide paraquat, we studied A549 cell line as a model for alveolar type II cells.

A549 cells exposed to the herbicide, showed an irreversible, dose-dependent loss of phalloidin-labelled microfilaments. After 1 hr treatment with paraquat 80 μ M actin filaments decrease was evident in 30% of the cells, and it became complete within 24 hrs.

Next we have investigated the actin levels in control and paraquat treated cells by immunoblotting technique. Cellular extracts, separated by SDS gel electrophoresis, were transferred to a nitrocellulose membrane and immunostained with monoclonal anti-actin antibody. Densitometric analysis of the blotting showed that actin level was 80% higher in paraquat treated cells than in control cells. However actin biosynthesis determined by 35 S-Methionine incorporation, was decreased after incubation with paraquat. Further studies are in progress to understand which step of actin turnover is affected by paraquat in A549 cells.

Tu-14 TWO TYPES OF MICROTUBULES IN THE FROG URINARY BLADDER EPITHELIUM STIMULATED BY VASOPRESSIN

J.J.Komissarchik and E.S.Snigirevskaya

Institute of Cytology of the Russian Academy of Sciences, St.Petersburg, Russia

Water permeability of the frog urinary bladder as we as other tight epithelia is regulated by antidiuretic hormone, vasopressin. Changes in the cytoskeleton of granular cells of the frog urinary bladder under conditions of low and vasopressin-induced water permeability were investigated. Using a conventional electron microscopical preparation with glutaraldehyde/osmium fixation we found only the usual type of microtubules in granular cells of both resting and stimulated bladders. These microtubules, 20 nm diameter, were associated with centrioles and Golgi complexes. Under the high permeability conditions the large vesicles appear in granular cells. The 20-nm microtubules and microfilaments were connected to the membrane of these vesicles. The second type of "thick" microtubules was found in granular cells of the stimulated bladder after the fixation with glutaraldehyde without osmium postfixation or freeze-substitution. The tubulin nature of these "thick" microtubules was identified immunocytochemically. In addition, tubulin was present in the granules with crystalline content.

Tu-16 COEXISTENCE OF CYTOKERATINS AND VIMENTIN IN THE PYRIFORM CELLS OF THE OVARIAN FOLLICLE OF THE LIZARD *Podarcis sicula*

M.G. Maurizii, C.Taddei.

Dipartimento di Biologia Evoluzionistica Sperimentale
Università di Bologna - Italy

The ovarian follicle of the lizards is interesting because, during most of the previtellogenetic growth, contains peculiar cells called for their shape "pyriform cells" (cfr. Taddei and Andreuccetti, 1990). These cells are connected to the oocyte through cytoplasmic bridges and are regularly distributed in the follicular epithelium between small and intermediate follicle cells. Furthermore it has been clarified that the pyriform cells differentiate from the small follicle cells after their heterologous fusion with the oocyte and that the intermediate cells represent a step in this differentiation.

For better clarify the structure and function of this polymorphic follicular epithelium we have studied the immunolocalization of intermediate filament proteins (cytokeratin and vimentin) in the different cells.

The cytokeratin, while is absent in the small follicle cells, is present in the cells (intermediate and pyriform) connected to the oocyte by an intercellular bridge. In particular in the pyriform cells this protein is mainly localized in their apex pointed toward the oocyte surface and forms a network which, through the intercellular bridge, crosses the zona pellucida and appears in continuity with an oocyte cortical ring of cytokeratin.

The vimentin instead is present in the cytoplasm of all the cells constituting the follicular epithelium. In the pyriform cells is mostly localized in their basal pole facing the ovarian follicle surface and never in the cytoplasmic bridges connecting these cells to the oocyte.

The presence of vimentin in all the follicular cells can be related to the mesenchymal origin of these cells; the coexistence of cytokeratin only in intermediate and pyriform cells can be ascribed to their connection to the oocyte via intercellular bridges and further evidences a functional integration between these follicle cells and the growing oocyte.

Tu-17

**TUBULIN-ACTIN INTERACTIONS
IN FISSION YEAST**

J.W. Cope and J.S. Hyams.

Department of Biology, University College London,
Gower Street, London, WC1E 6BT

We have used low temperature to depolymerise the cytoplasmic microtubules of the fission yeasts *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus var versatilis*. Exponentially growing wild type cells were rapidly cooled to 1°C. The interphase microtubule array that extends between the two cell poles (Hagan and Hyams, *J. Cell Sci.* **89**, 343-357, 1988), was depolymerised by 40 min. When cells were returned to 30°C, microtubules started to reappear by 2 min and were fully reestablished by 5-10 min. We investigated whether microtubule depolymerisation affected the distribution of F-actin which in fission yeast cells is normally associated with the growing tip(s) (Marks and Hyams, *Eur. J. Cell Biol.* **39**, 27-32, 1985). Following cold treatment, actin was delocalised except in a small percentage of cells (~2%) where a ring of F-actin was associated with the nucleus. The restoration of actin to the cell tips was coincident with the reinitiation of microtubules (2 min at 30°C). These experiments were repeated in the mutant *ccl25.22* following arrest at the G2/M boundary of the cell cycle for two generations. Microtubules were again completely depolymerised by low temperature, and the actin displaced from the cell tips but cells having actin associated with the nucleus now accounted for about 20% of population. As with wild type cells, these rearrangements of F-actin were reversed 2 min after return to 30°C. We speculate that the tubulin-actin interactions in fission yeast are subject to cell cycle control.

Tu-19

**PLECTIN IS PHOSPHORYLATED BY p34^{cdc2}
KINASE AT ITS C-TERMINAL DOMAIN**

N. Malecz, R. Foisner, G. Wiche

Institute of Biochemistry and Molecular Cell Biology,
Biocenter, University of Vienna, 1030 Vienna, Austria

Plectin, is an abundant intermediate filament associated protein (IFAP) of high mol wt. Similar to other cytoskeletal elements intermediate filament (IF) networks undergo a dramatic rearrangement during mitosis. Phosphorylation of IF subunit proteins by p34^{cdc2} protein kinase has been suggested as one of the molecular mechanisms responsible for this rearrangement. Here we show that plectin is phosphorylated when incubated with mitotic CHO cell lysates. One of the kinases involved in mitotic phosphorylation has been identified as p34^{cdc2} protein kinase by protein p13^{suc1} depletion assays and specific immunoprecipitation of the kinase activity. 2-dimensional peptide mapping of phosphorylated plectin using different kinases showed that the phosphorylation site of p34^{cdc2} kinase is distinct from those of protein kinases A and C; and that it is restricted to a small region of the molecule. Sequence analysis of plectin cDNA indicated three consensus sites for p34^{cdc2} kinase, one residing at the 5' end of plectin's rod domain and two in the C-terminal domain. Using recombinant plectin mutant proteins expressed in bacteria as *in vitro* substrates, the location of the p34^{cdc2} specific phosphorylation site could be narrowed down to the C-terminal domain of the protein, containing the last of plectin's repeats and the tail. 2-dimensional peptide mapping of repeat 6, the tail, as well as intact plectin (all phosphorylated by p34^{cdc2}) confined the phosphorylation site to repeat 6; phosphorylation of the tail was probably unspecific. Using cDNA constructs encoding either one of the two proposed phosphorylation sites within repeat 6 led to the conclusion that only one of these sites served as a target site for p34^{cdc2} kinase. Phosphorylation of plectin by p34^{cdc2} kinase may thus be involved in the profound structural rearrangement of IF networks observable during mitosis.

Tu-18

**Genetic and molecular analysis of the
microtubule function in Drosophila**

Máthé, E., Jósvay, K. and Szabad, J.

Albert Szent-Györgyi Medical University,
Department of Biology,
Szeged, Somogyi B. u.4, H-6720, Hungary

Our strategy of genetic and molecular analysis of the microtubule function is to identify genes encoding tubulins and/or microtubule-associated proteins by isolating mutant alleles that affect the function of the gene product.

We have isolated gain- and loss of function mutant alleles of the *Tomaj*-gene. Performing the molecular analysis we demonstrate the *Tomaj*-gene encodes the alpha 4-tubulin.

The gain of function nature of the *Tomaj*-mutant alleles are due to frame-shift mutations and they encode truncated proteins which lack the C-terminal domain.

The genetic and immunohistochemical analysis of the *Tomaj*-gene demonstrates that this gene is expressed only in the female germ-line and the alpha 4-tubulin is required for the oocyte meiosis and embryonic cleavage divisions.

Tu-20

**LIGHT-DEPENDENT TRANSLOCATIONS OF
MITOCHONDRIA AND ER CISTERNAE IN
PHOTORECEPTOR CELLS OF THE
GRASSHOPPER *SCHISTOCERCA GREGARIA*
OCCUR ALONG ACTIN FILAMENTS**

K. Stürmer, O. Baumann, B. Walz

Institut für Zoologie, Universität Regensburg, Universitätsstr. 31,
D-93040 Regensburg, Germany

Light dependent positioning of cell organelles are a well known phenomenon in photoreceptor cells of arthropods. In photoreceptor cells of the grasshopper *Schistocerca gregaria* both, mitochondria and ER-cisternae show antagonistic, radial movements when stimulated by light. In dark-adapted photoreceptor cells, large submicrovillar cisternae of the ER are positioned next to the photoreceptive microvilli. After illumination the ER cisternae are translocated into other cellular areas, whereas numerous mitochondria aggregate closely at the microvilli. We have studied the involvement of cytoskeletal elements in these light-dependent translocation processes.

Electron microscopical examinations visualize bundles of filaments lying in close association to the translocated organelles and lining the track of their movement. Fluorescent phallotoxins, specific probes for F-actin, bind to the submicrovillar area. Similarly, in immunogold studies, monoclonal anti-actin antibodies bind to filamentous structures in this cell area. The submicrovillar area lacks microtubules in both chemically fixed, and high pressure frozen specimens. Finally, depolymerization of the submicrovillar actin filaments by cytochalasin B blocked the light-dependent translocation of both mitochondria and ER cisternae towards the rhabdom. These results indicate that actin filaments are involved in the light-dependent translocation of mitochondria and ER cisternae.

Supported by the DFG (SFB, I1 to B.W. and Ba 1284/1-1 to O.B.)

Tu-21 COMPARISON OF THREE ACTIN cDNAs FROM THE FLATWORM *DIPHYLLOBOTRHIUM DENDRITICUM* (CESTODA)

M.H. Wahlberg, K.A. Karlstedt and G.I.L. Paatero

Åbo Akademi University, Department of Biology, Artillerig. 6, FIN-20520 Åbo, Finland

Actin is a highly conserved protein that plays a fundamental role in many diverse and dynamic cellular processes. In most organisms actin is encoded by multiple non-allelic genes, and the purpose of this study is to examine three members of the actin multigene family of the tapeworm *Diphyllobothrium dendriticum*.

When screening a cDNA library of *D. dendriticum* (UniZap XR, Stratagene) with a human γ -actin probe, several positive clones were found. Three of these, *Didact1*, *Didact2* and *Didact3*, have been completely sequenced and the results show that the deduced actin proteins are 376 (*Didact1* and *Didact2*) and 377 (*Didact3*) amino acids long. The identity percentage when comparing the coding regions of the *Didact1* and the *Didact2* nucleic acids is 95.8, whereas the deduced amino acid sequences are identical except for amino acid 122, which is glutamine in *Didact1* and arginine in *Didact2*.

Didact3, on the other hand, varies to a greater extent from the other two actins. The coding region of the *Didact3* nucleic acid is only 83.8 % identical with *Didact1* and 84.2 % identical with *Didact2*. On the protein level the identity percentages are 92.3 and 92.0, respectively. The differences could indicate functional dissimilarities. The 5'- and 3'-non-coding regions of the three actin cDNAs are 37.4-48.0 % identical.

Tu-22 PRESSURIZATION OF CHONDROCYTES IN THE PRESENCE OF TAXOL AND NOCODAZOLE
M.O. Jortikka, M.J. Lammi, J.J. Parkkinen*, R.I. Inkinen, T. Häkkinen, H.J. Helminen and M.I. Tammi.
Departments of Anatomy and Pathology*, University of Kuopio, Finland.

We have shown earlier that continuous high hydrostatic pressure decreases [35 S]sulfate incorporation of chondrocytes, representing proteoglycan (PG) synthesis, simultaneously with reorganization of microtubules (MTs) and alterations in the Golgi apparatus and stress fibers. Therefore, we examined whether a MT stabilization with taxol would prevent the decrease in PG synthesis induced by the hydrostatic pressure.

Nocodazole and taxol were added into confluent chondrocyte cultures, and cultures were exposed to hydrostatic pressure (5-30 MPa 20 h in the presence of 10 μ Ci/ml [35 S]sulfate) in a special apparatus. After loading, nocodazole- and taxol-treated cells on glass coverslips were fixed and stained for Golgi apparatus by TRITC-conjugated WGA, and immunostained for the MTs by a monoclonal antibody against α -tubulin.

Taxol decreased [35 S] sulfate incorporation rate only minimally, while nocodazole caused a 40% reduction. Cyclic 0.5 Hz loading at 5 MPa induced an increase in [35 S]sulfate incorporation rate, however, in the presence of nocodazole, the stimulation was lost. A continuous 30 MPa hydrostatic loading decreased the [35 S]sulfate incorporation by 35%, and taxol and nocodazole further decreased it into levels 40% and 20% of the control, respectively. Thus, stabilization of MTs with taxol could not prevent the pressure-induced reduction of [35 S]sulfate incorporation. In control chondrocytes, the MTs radiated from the MT organizing center to the plasma membrane showing the typical staining, and the Golgi was localized as a juxtanuclear cap. Taxol treatment caused hyperpolymerization of the MTs in the cytoplasm and fragmentation of the Golgi. When a 30 MPa hydrostatic pressure was applied to the taxol-treated cells, the effect of taxol on MT staining pattern almost disappeared, and the Golgi apparatus was packed into a clump. In conclusion, the reorganization of MTs by the high 30 MPa hydrostatic pressure may be associated with PG secretion/synthesis. However, there is probably an MT-independent system affected by the high pressure, too.

Tu-23 ANALYSIS OF CARTILAGE SPECIFIC TRANSCRIPTS DURING CHONDROGENESIS
S. Muratoglu, Cs. Bachrati, F. Deák, I. Kiss, Institute of Biochemistry, BRC, POB 521, Szeged, Hungary 6701

RNA was isolated from chondrocytes of different developmental stages: (1) Mesenchyme cells from limb buds of stage 25 (day 4.5) chicken embryos plated at high density to stimulate chondrogenic differentiation (HDM cultures). (2) Proliferative chondrocytes from sterna of day 14.5 chicken embryos. (3) Resting chondrocytes from articular cartilage and the xyphoid process of sterna of juvenile chicks.

Expression of genes for collagen II, link protein (LP) and cartilage matrix protein (CMP) was monitored in Northern hybridization. mRNA-s for collagen II and LP were detectable from the first day in HDM culture and their amount increased by a steady, but low rate up to day 6. CMP mRNA was first detectable only on day 4 of HDM culture but showed a steep increase in amount. This observation suggests that the pattern of gene expression in vitro recapitulates the events observed *in situ* in limb buds.

Besides the 3.4 kb mRNA for CMP described previously, another transcript of 2.1 kb was observed in proliferative chondrocytes, the amount of which was relatively higher in the cartilage of juvenile chicks. The stage specific variation in the usage of polyadenylation sites in generating those transcripts and in alternative splicing of the 5' untranslated region of LP mRNA is under investigation.

CANCELLED

THE DISTRIBUTION OF GAMMA-TUBULIN IN
GERM CELLS OF INSECTS

Tu-25 K. W. Wolf, Inst. Biol., Med. Univ.
Lübeck, Ratzeburger Allee 160,
23538 Lübeck, Germany

Spindle structure in male meiosis of Lepidoptera is remarkable in that most spindle microtubules (MTs) end halfway between the equatorial plate and the centrosomes in metaphase (1). These MTs do not have contact with MT-organizing centres in the form of the centrosomes and may have an inherent stability. What renders them stable? A newly discovered tubulin isoform, gamma-tubulin (2), is contained within the centrosomes of a wide variety of cell types and probably plays a role in the nucleation of MTs. Gamma tubulin was also detected in the midbody of mammalian cells, a system of relatively stable MTs (3). In the present study, spermatocytes of the Mediterranean mealmoth, *Ephestia kuhniella* (Pyralidae, Lepidoptera) were probed with a monoclonal antibody against gamma-tubulin (2). The antibody detected - as expected - the centrosomes, but there was also faint staining of the non-centrosomal MTs of metaphase spermatocytes. Thus, gamma-tubulin may be involved in conferring stability to these MTs.

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The antibody against gamma-tubulin was a gift of Dr. H. Joshi (Emory Univ. Atlanta, U.S.A.). Supported by the "Deutsche Forschungsgemeinschaft" (Wo 394/6-1).

INHIBITION OF HEPATOCYTIC AUTOPHAGY
AND SELECTIVE DISRUPTION OF THE
CYTOSKELETON BY OKADAIC ACID

Tu-27 Henrietta Blankson, Ingunn Holen, Per O. Seglen
Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway

Autophagy, a non-selective process by which cells sequester and degrade portions of their cytoplasm, was measured as the sequestration of endogenous lactate dehydrogenase. It was shown that the protein phosphatase inhibitor okadaic acid (30nM) completely inhibited autophagy in isolated rat hepatocytes when administered for more than 30 min. Vinblastine (10µM) and cytochalasin D (10µM), drugs that induce depolymerization of microtubules and microfilaments, respectively, had only moderate effects on autophagic sequestration even if given for up to 150 min. Immunofluorescence staining with an antibody against an intermediate filament protein, 55KD cytokeratin (CK55), showed that while cytokeratin in control cells consisted of a fine network surrounding the nucleus, the cytokeratin filaments in okadaic acid treated cells were disrupted. Instead of a network, small spherical aggregates were seen spread through the cytoplasm. Indirect immunofluorescence staining of microtubules using monoclonal antibodies against α- and β-tubulin, and staining of microfilaments using rhodamine-phalloidin, showed no noticeable effect of okadaic acid on these cytoskeletal elements. These results indicate a possible role for cytokeratin intermediate filaments in the autophagic process.

Reorganization of cytoskeleton in polykaryons obtained by cell fusion

Tu-26 G.E.Onishchenko, N.O.Yarovaya
Department of Cytology and Histology,
Biological Faculty, Moscow State University,
Moscow 119899, Russia

In PEG-fused cells it was observed that firstly disordered microtubular (MT) network (2 hrs post-fusion) was replaced by a radial MT system (5 hrs post-fusion), then disordered MT pattern was found again (6 hrs post-fusion), but later reappearance of the radial MT system occurred (12-18 hrs post-fusion). Vimentin filaments formed the radial system only once when MT were organized in a radial way for the second time. Such reorganization of MT system was found also in cytochalasin B (CB)-treated polykaryons, but vimentin filaments in that case did not form radial system and were gradually forced out toward the cell periphery. Electron microscopic studies confirmed the absence of the united cell centre early after the cell fusion (2 hrs) both in control and CB-treated cells and the presence of this one in 5 hrs after the cell fusion, but in control cells it contained all centrioles, while in CB-treated polykaryons centrioles were not grouped together and were located near individual nuclei. Shift of centrioles toward the cell centre in CB-treated cells occurred later: in 18 hrs after the cell fusion the structure of the united cell centre was similar in control and CB-treated cells. These data allow to suppose that there is a correspondence between the variant of cytoskeleton organization and the structure and functioning of cell centre.

ACTIN ORGANIZATION IN YARROWIA
LIPOLYTICA DURING THE CELL CYCLE

Tu-28 I. Hönes^a, M. Havelkova^b, K.J. Böhm^a and E. Unger^a
^a Dept. Molecular Cytology, Institute of Molecular Biotechnology, Jena, Germany, ^b Dept. Biology, Medical Faculty, Masaryk University, Brno, Czech Republic

Yarrowia lipolytica is a dimorphic yeast that has a cell cycle similar to *Candida albicans*. In YEPD medium a part of cells grow as budding yeast and another part as hyphae. The morphology of both development stages is not well characterized. The aim of this study was to extend the knowledge on morphology of *Yarrowia lipolytica* by determining the distribution of actin, which is known to play a key role in cell shaping and polarization in numerous different cells.

For visualization of actin, budding and hyphal cells were stained with rhodamine-phalloidin and examined in a fluorescence microscope. In addition, the same samples were treated with DAPI to characterize the cell cycle stage and with calcofluor white to distinguish between mother and daughter cells by visualization of chitin (mainly located in the bud scars and in the septa). In this way, each cell could be assigned to a particular point in the cell cycle.

Actin granules were distributed beneath the plasma membrane in budding G1-cells, before starting cell growth. Then actin was concentrated at the sites where the bud will be formed. During budding, actin dots were arranged in two lines at the neck between mother and daughter cell. During bud growth actin dots were located solely in the bud (S/G2-phase). The most large buds contained both actin granules and fibres (G2 phase).

For G1-hyphal cells actin granules were also found to be localized beneath the plasma membrane. During hyphal growth, the majority of actin dots clustered at the hyphal apex and at septum forming sites.

From these results we conclude that actin is required for determination of the site of bud formation and for cell growth also in *Yarrowia*.

Tu-29 CATECHOLAMINE AND SEROTONIN BINDING TO CYTOPLASMIC ACTIN CONSTITUTES FOR A PART OF THE 'SEROTONIN BINDING PROTEINS'

J. Pinyerlen¹, C. Velez Pardo², M. Jimenez Del Rio², G. Vauquelin¹, G. Ebinger³ and W. De Potter¹. Neuropharmacology¹, University of Antwerp (UIA) and Protein Chemistry² and Neurology³, Free University of Brussels (VUB).

Binding of [³H]serotonin, [³H]dopamine and [³H]noradrenaline to bovine brain cytoplasmic actin is increased by Fe²⁺ but not by Fe³⁺. Fe²⁺ is known to generate radical oxygen species such as superoxide radicals. They are well known to oxidise monoamines and the oxidation products are recognised to bind covalently to external nucleophiles such as sulphydryl groups on proteins. The occurrence of such covalent binding mechanism in the case of rabbit skeletal muscle actin was suggested before e.g. by the inability of a large excess (1 mM) of serotonin or dopamine to diminish the binding when added to a pre-equilibrated mixture of actin, 0.1 mM Fe²⁺ and 0.2 μM radioligand. Serotonin and catecholamines bind covalently to cytoplasmic actin under the very same conditions as to the so-called 'serotonin binding proteins' (SBP), consisting of two major proteins (56 and 45 kDa). Up to 60% of the binding of serotonin or catecholamines to actin can be inhibited by certain vinalkaloids (e.g. vinblastine). For 'partially purified' SBP, this inhibition is up to 40%. Since SBP preparations are enriched for cytoplasmic actin, and we observed that radioactive labelling coincides mainly with this cytoplasmic actin on SDS-PAGE, we suggest that cytoplasmic actin is one of the two major SBP.

Tu-31 TWO SUBFRACTIONS OF THE NUCLEOLAR PROTEIN FIBRILLARIN ACCOUNT FOR ITS DIFFERENTIAL PRESENCE IN PLANT NUCLEOLI DEPENDING ON NUCLEOLAR ACTIVITY

F.J. Medina and A. Cerdido

Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006 Madrid, Spain

Fibrillarin is a conserved constituent of the snoRNP particles which acts in the first cleavage of pre-rRNA. It has been reported that fibrillarin localizes to the dense fibrillar component (DFC) of the nucleolus, even in stages typical of nucleolar inactivation (1,2). We have identified fibrillarin in onion cells, by means of a human autoimmune serum, as a 37 kDa protein, and quantitatively detected it in the transition area fibrillar centers-DFC (the putative site of nucleolar transcription), and in the proximal zone of the DFC, but not in the distal DFC zone. The quantitative levels of fibrillarin in the nucleolus were shown to double from G1 to the G2 phase of the cell cycle, in relation to the increase in nucleolar activity. Fractionation of nuclei and Western blot analysis allowed the discrimination of two fractions of fibrillarin, one of them soluble in low ionic strength/EDTA buffer, associated to the nuclear RNP fraction, and the other insoluble, only extractable with 7 M urea and high salt. The nuclear chromatin fraction did not contain fibrillarin. From this result, we suggest that the soluble form is active in its role in pre-rRNA processing, while the insoluble form is inactive in this function, and may play a structural role in the nucleolar matrix and/or is being stored for further use. This is consistent with the finding that undifferentiated, rapidly growing meristematic cells are more enriched in soluble fibrillarin than differentiated parenchymatic cells.

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Tu-30 MICROTUBULE-MEDIATED MOVEMENT OF MEIOTIC NUCLEI IN SCHIZOSACCHAROMYCES VERSATILIS

A. Svoboda^a, J. Bahler^b, K. Kohli^b

^aDepartment of Biology, Faculty of Medicine, Masaryk University, 662 43 Brno, Czech Republic. ^bDepartment of General Microbiology, University of Bern, CH-3012 Bern, Switzerland

In the homothallic *Schizosaccharomyces versatilis* strain, the fused nuclei, retaining their elongated (horse-tail) shape during pre-meiotic interphase and meiotic prophase, underwent several changes in position between the poles of the zygote and, eventually, divided in the central position. Metaphase II occurred 20 to 30 min later, followed by postmeiotic mitosis after 30 to 60 min. We observed that the horse-tail nucleus was attached by its SPB to a junction of two arrays of cytoplasmic microtubules extended along the whole length of the zygote. Each array of microtubules originated from one mating-activated partner. Shortening and extending of the respective microtubular arrays moved the junction with the attached nucleus. Prior to spindle formation, the cytoplasmic microtubules disappeared to reappear later as astral microtubules emanating from the SPB. A similar microtubular dynamics was seen in the following stages of meiosis and postmeiotic mitosis. The arrays of astral microtubules were also found in spores.

Tu-32 SUPPRESSION OF VILLIN EXPRESSION BY ANTISENSE RNA IMPAIRS BRUSH BORDER ASSEMBLY AND SUCRASE-ISOMALTASE APICAL INSERTION IN POLARIZED EPITHELIAL INTESTINAL CELLS

M.A. Costa de Beauregard, E. Pringault, S. Robine and D. Louvard

Institut Pasteur, 25 rue du Dr. Roux 75015 Paris, France.

We have used an antisense RNA strategy to investigate the role of the actin-associated protein, villin, in the brush-border morphogenesis of human intestinal CaCO₂ cells.

Stable expression of a cDNA encoding antisense villin RNA resulted in the permanent down-regulation of the endogenous villin message. The absence or low level of villin in these cells strongly modified the apical cell surface, which displayed a small number of dispersed and short microvilli. F-actin bundles were not observed in the rudimentary microvilli, whereas F-actin stress fibers appeared in the cytoplasm of the villin antisense cells.

Ultrastructural and immunolocalization studies (of the tight junction protein ZO1, the basolateral marker 120kD and the apical hydrolase dipeptidyl-peptidase IV) showed that epithelial cell polarity was largely maintained. However, in contrast to brush border markers such as dipeptidyl-peptidase IV, neutral amino peptidase and neutral endo peptidase, the apical localization of sucrase-isomaltase was specifically impaired.

Super-transfection of the villin antisense-expressing cell line with a cDNA encoding a partial sense villin RNA restored both brush-border assembly and sucrase-isomaltase apical expression. We suggest that brush border morphogenesis may be important for the trafficking of certain apical proteins.

Characterization of the binding of calmodulin to non-erythrocyte spectrin

Tu-33 Johanna Björk, Susanne Lundberg and Lars Backman
Departement of Biochemistry, University of Umeå,
S-901 87 Umeå, Sweden.

When immobilized on a PVDF membrane both brain and erythrocyte spectrin bound calmodulin in a calcium dependent manner. The affinity of the non-erythroid spectrin was much greater than that of the erythroid form. Using equilibrium partition the interaction was characterized further, and in the presence of calcium the partition behaviour of calmodulin was affected by both spectrins, though brain spectrin caused a much larger change in partition. It was evident, in both cases, that the observed partition behaviour of calmodulin was due to complex formation with spectrin. The equilibrium partition data was analyzed and the amount of bound calmodulin in the solid-phase assay was quantified, indicating the presence of two binding sites for calmodulin on brain spectrin; one high-affinity site characterized by a dissociation constant of about 0.3 μM and a much weaker (>0.3 mM) second binding site. The presence of two binding sites was further substantiated by the observation that truncated recombinant spectrin fusion proteins comprising either the middle part or the C-terminal of non-erythroid α-spectrin bound calmodulin.

MICROTUBULE AND CHROMATIN CONFIGURATION IN GROWING PIG OOCYTES: EFFECTS OF PHOSPHATASE INHIBITORS

Tu-35 J. Rozinek^a, J. Petr^a, F. Jílek^b
^aRes. Inst. Anim. Prod., ^bUniversity of Agriculture, Praha, Czech Republic

Three distinct chromatin (Cr) configurations and two types of microtubule (MT) arrangement were observed during the growth period in pig oocytes. The oocyte with an internal diameter ranging from 60 to 90 μm exhibited diffuse, fibrous Cr within the nucleus and MT formed a filamentous network with a marked perinuclear array. Cr was more condensed in larger oocytes (diameter 100–110 μm) and MT formed a "sponge-like" structure. This type of MT arrangement is maintained in fully grown oocytes (diameter 120 μm) in which Cr is condensed around the nucleolus in the shape of a ring or a horseshoe.

After meiosis resumption in fully grown oocytes the typical sequence of changes in Cr configuration and MT arrangement was seen. Condensed chromosomes formed a metaphase I plate and MT formed a meiotic spindle. Such oocytes formed numerous cytoplasmic asters in response to the taxol treatment. The induction of meiosis resumption by caliculin A (CAL) (100 Nm) occurred without establishing M-phase MT arrangement. Similarly, okadaic acid (OA) (1 μM) induced meiosis resumption but the M-phase MT arrangement is partially established.

Growing oocytes smaller than 80 μm did not resume meiosis spontaneously and their maturation cannot be induced by treatment with CAL or OA. Despite partial condensation of Cr within the nucleus, their MT arrangement is not influenced after CAL or OA treatment. In larger oocytes meiosis resumption is significantly elevated after CAL or OA treatment but their MT arrangement remained unchanged.

SPATIAL NUCLEAR ORGANIZATION OF VIMENTIN EPITOPE-CONTAINING CHROMATIN

Tu-34 H. Fidlerová^a, A. Dahlström^b, V. Sovová^a, M. Nepraš^c, M. Šeps^c, V. Fidler^d, P. Engst^e, P. Kubát^e, V. Viklický^a and G. Levan^e

^a Institute of Molecular Genetics and ^e J. Heyrovský Institute of Physical Chemistry, AS CR, Prague, CR; ^b University of Göteborg, Göteborg, Sweden; ^c University of Pardubice, Pardubice, CR; ^d Charles University, Prague, CR

Different spatial nuclear compartments of the early and late replicating chromatin were immunovisualized by monoclonal antibody VI-01 and 3D-confocal microscopy in rat fibroblastic 49F cells. A photocrosslinking approach allowed detection of the VI-01-reactive vimentin epitope in bromodeoxyuridine-substituted chromatin only (Hereditas 117:265–273, 1992).

Using homobifunctional photocrosslinker 4,4'-bis azido-2,2'-disulfostilbene and laser-induced photocrosslinking *in situ*, a penetration of vimentin filaments to the nucleus was immunovisualized.

Microtubule-organizing centers of interphase and mitotic melanophores of *Xenopus laevis* *in vivo*

Tu-36 E.N. Nikeryasova^a, G.E. Onishchenko^a, K.A. Rubina^a, S.M. Starodubov^b

^a Department of Cytology and Histology, ^b Department of Embriology, Moscow State University, Moscow 119899, Russia.

The following data were obtained as a result of an electron-microscopy study of serial ultrathin sections of interphase and mitotic melanophores of *Xenopus laevis* larva at 51–53 stage of development.

In interphase cells centrosome contains two or numerous centrioles, surrounded by pericentriolar material in the form of fibrillar material or satellites or foci of convergence of microtubules. MTOC (microtubule-organizing center), including centrosome, has a different structure in melanophores in dispersed and aggregated state. In the first case melanosomes are situated in the immediate vicinity of the centrosome; in the second – MTOC consists of three zones: centrosome, centrosphere and outlying radial arrangement of microtubules and inclusions. In bipolar and multipolar spindles of mitotic melanophores the same zonality of MTOC is observed as in aggregated melanophores, but fibrillar material forms mitotic halo around the centrosomes.

Melanophores, being able to hormone-induced translocation of melanosomes within aggregation-dispersion processes, as well as to chromosome movements in mitosis, are a convenient model for studying MTOC changes and corresponding types of intracellular transport.

THE ROLE OF ACTOMYOSIN IN THE PLASMA MEMBRANE

L. Mirčevová

Tu-37
Institute of Hematology and Blood Transfusion, Prague, Czech Republic

The function of actomyosin in the plasma membrane of human erythrocytes (ery) was studied. The actomyosin, a contractile membrane protein, has Mg^{2+} -ATPase activity (AM-ATPase), which is strongly dependent on a Ca^{2+} concentration. The AM-ATPase activity is influenced not only by compounds affecting smooth muscles but also by many others. The compounds, which change the AM-ATPase activity, change also the shape or even the size of ery, and the passive permeability of K^+ in ery. After treatment of ery with F⁺, the correlation exists between inhibition of their deformability and AM-ATPase activity. Denaturation of AM-ATPase, irreversible changes of ery shape and irreversible decrease of ery deformability begin at the same temperature of 48°C. Inhibitors of AM-ATPase activity, which were tested on polymorphonuclear leukocytes, inhibited their phagocytic activity, and damaged their nuclei.

We suppose that under normal physiological circumstances actomyosin causes a slight membrane contraction, which conditions the typical ery shape. The state of the membrane actomyosin influences the deformability of ery, the state of the calcium-activated K channel, and the phagocytosis. Very probably actomyosin is also present in membrane of nuclei, and conditions some of its functions.

UNCONVENTIONAL MYOSINS IN THE DEVELOPING CHICK BRAIN

Tu-39Anthony Lodge and Diana Moss

Department of Human Anatomy and Cell Biology, The University of Liverpool, PO Box 147, Liverpool L69 3BX, United Kingdom.

Myosins both conventional and unconventional are likely to play an important role in the actin-based motile activities of neurons and their growth cones. These may include actin-dependent axoplasmic transport, retrograde actin flow within growth cone lamellipodia, and the formation of filopodia during cell-matrix interactions. The coordination of these events is essential for the pathfinding abilities of growth cones during development. We have identified four proteins in embryonic chick brain which will bind to actin filaments in an ATP-dependent manner, possess anti-myosin immunoreactivity and can bind calmodulin in the absence of calcium ions. The M_r's of the heavy chains varies between 110 and 120 kDa, suggesting that they may belong to the class I myosins. Partial amino acid sequence data have been obtained from tryptic peptides of the major 120 kDa protein and oligonucleotide primers corresponding to two hexapeptides have been designed. In combination with four primers corresponding to amino acid motifs conserved in all classes of myosin, we have used the polymerase chain reaction to amplify a number of cDNA clones which are being characterised by sequencing. We will use these probes to study myosin function *in vivo*.

DYNAMICS OF THE INTRACELLULAR CALCIUM CONCENTRATION AND MICROTUBULES IN A23187-TREATED PK CELLS.

I.B. Alieva and I.A. Vorobjev

A.N. Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow, Russia.

Addition of $10 \mu g/ml$ of A23187 gave an increase of $[Ca^{++}]$ in cytosol more than 10 times. The maximum $[Ca^{++}]$ was observed in 1 - 2 min after introduction of the drug. Later on $[Ca^{++}]$ was gradually decreased and after 30 min incubation with A23187 $[Ca^{++}]$ was 3-5 times above the normal level. Immunofluorescence study didn't show any alterations in the microtubule system of interphase cells after 1-30 min treatment. In mitotic cells spindle morphology remained normal up to 30 min. In the presence of A23187 cells continue dividing for 30 min. However, in metaphase cells we observed rapid shrinkage of pole-to-pole distance in 1 min after introduction of the drug. Electron microscopy demonstrated that in interphase cells 3 min after introduction of the drug more than 50% of maternal centrioles were oriented perpendicular to the substrate surface.

We conclude that increase in cytosolic $[Ca^{++}]$ induce immediate answer in the centrosome, while have little or no effect on cytoplasmic and spindle microtubules.

PROMOTER ELEMENTS AND TRANSCRIPTIONAL CONTROL OF THE CHICKEN β -TROPOMYOSIN GENE**Tu-40**M. Toutant, M. Fiszman and M. Lemonnier

Unité de Biochimie, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris cedex 15, France

The chicken β tropomyosin (β TM) gene has two alternative transcription start sites (sk and nmCAP sites) which are used in muscle or non muscle tissues respectively. In order to understand the mechanisms involved in the tissue-specific and developmentally-regulated expression of the β TM gene, we have analyzed the 5' regions associated with each CAP site. Truncated regions 5' to the nmCAPsite were inserted upstream to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and these constructs were transfected into avian myogenic and non myogenic cells. The maximum transcription is driven by the CAT construct (-168/+216 nt) in all cell types (basal promoter), and this correlates with the accumulation of the endogenous β Tmn transcript in the same cells. Previous deletion analysis of the region 5' to the β TMsCAP site has indicated that 805 nt confer myotube-specific transcription. In this work, we characterize an enhancer element (-201/-68 nt) which contains an E box (-177), a CArG-like motif (-104) and a stretch of 7Cs (-147). Mutation of any of these motifs results in a decrease of the myotube-specific transcriptional activity. Electrophoretic mobility shift assays indicate that these *cis*-acting sequences specifically bind nuclear proteins. This enhancer functions in an orientation-dependent manner. Finally, we show that the 805 nt 5' to the β TMsCAP which promote maximum tissue-specific activity in avian myogenic cells only drive background levels of transcription in murine myogenic cells, in transient or in stable transfectants.

LOCALIZATION OF UBIQUITOUS AND NEURONAL KINESINS IN THE CENTRAL NERVOUS SYSTEM OF THE RAT

Tu-41 Francesca Navone, Giovanna Vignali, Giorgio Battaglia^a,

M. Teresa Sprocati and Ronald Vale* - CNR Center of

Cytopharmacology, Dept. of Medical Pharmacology, Univ. di Milano, 20129 Milano, Italy; * Dept. of Pharmacology, University of California, CA 94143, USA and ^aIst. Naz. Neurologico "C. Besta", 20133, Milano.

Ubiquitous and neuronal kinesins (uKHC and nKHC, respectively) are two isoforms of the conventional microtubule-based motor which differ dramatically in their tissue expression pattern. Whereas uKHC is expressed in all tissues, nKHC is expressed only in neurons (Niclas et al., 1994, *Neuron*, in press). In the present study we analysed the distribution of uKHC and of nKHC in the central nervous system of the rat by immunofluorescence using polyclonal antibodies that recognize specifically each kinesin. Immunoreactivity for both uKHC and nKHC shows a diffuse distribution in all regions of the gray and white matter examined. However, in addition to the uniform distribution, immunofluorescence for nKHC, but not for uKHC, is selectively concentrated in well defined populations of neurons. For example, nKHC immunoreactivity is particularly abundant in pyramidal cells of the V layer of the cerebral cortex, in neurons of some, but not all, thalamic nuclei and in numerous cells of the pallidum and of the substantia nigra. In the hippocampus, the cells of the hilus stand out due to the intense fluorescence of their perikarya. In the cerebellum, the expression level of nKHC is high in Purkinje cells and in some neurons of the deep cerebellar nuclei. When compared to the distribution of MAP2, a well known somatodendritic marker, nKHC immunofluorescence is present in MAP2-positive cell bodies and dendrites as well as MAP2-negative bundles of myelinated axons. Our observations indicate that uKHC and nKHC have different cellular distributions in the brain and suggest that nKHC is enriched in subsets of neurons which may share some common physiological properties.

DIFFERENTIAL EXPRESSION OF UBIQUITOUS AND NEURONAL KINESINS DURING NEURONAL DIFFERENTIATION OF HUMAN NEUROBLASTOMA CELLS

Tu-42 Giovanna Vignali, Joshua Niclas*, M. Teresa Sprocati,

Ronald D. Vale*, Carlo Sirtori and Francesca Navone. CNR Center of Cytopharmacology, Dept. of Med. Pharmacology, Univ. of Milano, 20129 Milano, Italy and ^aDept. of Pharmacology, Univ. of California, San Francisco, CA 94143, USA.

Kinesin is a microtubule-based motor protein involved in organelle transport in neuronal and non-neuronal cells. Although a single kinesin motor has been thought to serve all cell types, we have recently identified and characterized a second human kinesin heavy chain (KHC), which is expressed exclusively in neurons (1). This kinesin isoform neuronal (nKHC) kinesin, and it is different from the previously characterized kinesin, which is ubiquitously distributed (uKHC) (2). To investigate whether the two kinesin motors serve distinct functions in neuronal cells, we studied the expression of uKHC and nKHC during neuronal differentiation. The human neuroblastoma cell line SH-SY5Y shows morphological and functional neuronal differentiation when grown in the presence of retinoic acid (3). Using antibodies specific for uKHC and for nKHC we performed immunofluorescence and immunoblotting experiments to evaluate the expression of the two kinesin isoforms in neuroblastoma cells before and after neuronal differentiation. We found that, while the expression of uKHC is not affected by treatment with retinoic acid, the expression of neuronal kinesin is stimulated 4-5 fold during the acquisition of neuronal phenotype. Our results indicate that retinoic acid treatment induces a selective increase of nKHC, but not of uKHC, thus suggesting that nKHC may play a fundamental role in microtubule-based transport of cells undergoing neuronal differentiation.

References: 1) Niclas et al. (1994) *Neuron*, in press. 2) Navone et al. (1992) *J. Cell Biol.* 117: 1263-1275. 3) Giudici et al. (1992) *Eur. J. Cell Biol.* 58: 383-389. - G.V. is a recipient of a SOCREA fellowship.

XKLP2, A XENOPUS "KLP" LOCALIZED IN THE CENTROSOMES AND MITOTIC SPINDLE

Tu-43 H.Boleti, I. Vernos, E. Karsenti
EMBL, Meyerhofstrasse 1, Heidelberg, Germany

Classical and molecular genetics and sequencing of mutants in microtubule (MT) dependent processes in several organisms has uncovered a class of proteins containing regions with about 40% sequence identity to the Drosophila Kinesin heavy chain (KHC) motor domain. These proteins are termed Kinesin-like proteins (KLPs) and are involved in MT based motility processes during mitosis and meiosis as well as in vesicle and organelle transport.

We are studying KLPs in Xenopus. XKLP2 was cloned by PCR technology from a Xenopus oocyte cDNA library. Upon sequencing it was found to have 28-45% identity with sequences containing the motor domain of KHC and of other KLPs. Sequence comparison analysis indicated that XKLP2 is not the homolog of any previously identified KLP. Secondary structure prediction analysis suggested similar structural domain organization as the majority of identified KLPs: a globular domain at the NH₂-terminus (head), containing the putative "motor" region sequences and a long α -helical region (stalk) predicted to form coiled coils.

We have generated antibodies (Abs) directed to sequences from the C-terminal region of XKLP2. These Abs recognised in Western Blots a protein from Xenopus egg extracts with an apparent Mr of 160 Kda, about the same Mr calculated from the XKLP2 primary structure. XKLP2 was also detected by Western Blot analysis in a preparation of MT "motor" proteins, indicating that XKLP2 interacts with taxol stabilised MTs and is eluted from MTs by Mg-ATP.

Immunolocalization studies of XKLP2 in Xenopus eggs and the XL177 cultured cells showed that it is localized in the centrosomes, during interphase and prophase and on the spindle poles and spindle MTs during mitosis. Studies to elucidate the function of XKLP2 are under progress.

XKlp1, A XENOPUS KINESIN-LIKE PROTEIN ESSENTIAL FOR CYTOKINESIS

Tu-44 I. Vernos^a, J. Raats^b, J. Heasman^b, C. Wyllie^b and E. Karsenti^a

^aEMBL, Meyerhofstrasse 1, 69117 Germany; ^bWellcome CRC Institute, Tennis Court Road, CB2 1QR Cambridge, England. The kinesin family of microtubule motor molecules include an increasing number of proteins involved in organelle transport and different aspects of the meiotic and mitotic spindles organization and function.

We are presently cloning and characterizing kinesin-like proteins in Xenopus. XKlp1 cDNA has been obtained from a Xenopus oocyte cDNA and has been fully sequenced. The predicted protein contains a motor domain at the amino end with an overall identity of around 40% with the homologous region of the other members of the family. This domain is linked to a predicted globular tail domain by a stalk predicted to be part in coiled-coil interactions. This overall organization is similar to that of many kinesin-like proteins including kinesin itself.

We have produced two antibodies specific for XKlp1. On Western blots of high speed Xenopus egg supernatants, both antibodies recognize a single protein of Mr 150 Kda. This protein binds to taxol stabilized microtubules in the presence of AMP-PNP and apyrase and is released partially from them by addition of Mg-ATP, behaving in this assay like a motor protein.

We have analyzed the immunofluorescent pattern of wholemount stage 8 Xenopus embryos using an anti-tubulin and an anti-XKlp1 antibodies. XKlp1 is concentrated on the centrosome area during prophase and on the spindle in metaphase and early anaphase. At late anaphase it becomes progressively relocalized on the mid-zone where it forms a bulding ring throughout telophase. Finally the midbody is brightly stained.

To further determine the function of XKlp1, anti-sense oligonucleotides have been injected in Xenopus oocytes in order to deplete the system of endogenous XKlp1 mRNA. The resulting embryos show an arrest in cell cleavage while the nuclei are still able to divide. This data indicates that XKlp1 performs an essential role in cytokinesis.

Tu-45 PRIMARY STRUCTURE AND GENE ORGANIZATION OF AN UNCONVENTIONAL MINI-MYOSIN HEAVY CHAIN FROM THE GREEN ALGA ACETABULARIA

Oliver Vugrek and Diedrik Menzel

Max-Planck-Institut für Zellbiologie, Rosenthal, D-68526 Ladenburg, Germany

The giant unicellular green alga Acetabularia is well known for its multistriate type of cytoplasmic streaming. We have previously shown that movement of organelles proceeds along an extensive network of actin filament bundles but the molecular motor responsible for these movements has remained elusive. We have now been able to isolate and sequence a cDNA clone which codes for a unique new type of myosin heavy chain (MHC) of only 927 amino acids lacking most of the alpha-helical tail portion usually containing several calmodulin-binding sites in class I MHCs. We also provide the complete sequence of the corresponding gene including about 4.5kb of 5' upstream and 2.5kb 3' downstream regions. The gene is split into eight exons and stretches over about 16kb of genomic DNA. We have thus characterized the primary structure of a possible candidate for acto-myosin based organelle motility in Acetabularia.

Tu-46 MHC1emb: AN ADDITIONAL SLOW ISOFORM OF THE MYOSIN HEAVY CHAIN FAMILY

BRUSON A., RIZZI C.* ROSSINI K., SANDRI M. and CARRARO U.

C.N.R. Centro di Studio per la Biologia e Fisiopatologia Muscolare c/o Dip. Sci. Biomed. Sper. dell'Università' di Padova, Via Trieste 75, I-35121 Padova (Italy).

* Istituto di Chirurgia Plastica e Ricostruttiva, Via Giustiniani 2, Università' di Padova. Direttore Prof. Francesco Mazzoleni.

Myosin is an esopolyptide, which isoforms are segregated during development in different fiber types. Several isoforms in mammals (MHC emb, MHC neo, MHC 1, MHC 2A, MHC 2B, MHC 2/x/D/S) can be separated by SDS PAGE. The presence of an additional embryonic MHC1 isoform is suggested by rat and human studies. We confirm MHC1 heterogeneity by immunochemical analyses: a monoclonal anti-MHC1 antibody which selectively binds to the adult slow and B-cardiac MHC doesn't react with the MHC1 band present in SDS PAGE of MHC from rat embryonic muscle.

We purify MHC1 from Cardiac, Soleus and embryonic rat muscles by Electroendosmotic Preparative Gel Electrophoresis (Rizzi C. and Carraro U. Basic Appl. Myol. 1, 43-53, 1991), concentrating in the purified polypeptides by SDS protein precipitation technique (Sandri M., Rizzi C., Catani C. and Carraro U. Anal. Biochem. 213, 33-34, 1993).

Peptide mapping by chemical and enzymatic cleavage together with immunochemical analyses, suggest that the MHC1-emb has a peculiar aminoacid sequence.

Tu-47 LIGHT-INDUCED CHANGES OF cGMP IN STENTOR CILIATES

H. Fabczak*, M. Walerczyk*, S. Fabczak* and P.-S. Song^b

^aDepartment of Cell Biology, Institute of Experimental Biology, Warsaw, Poland; ^bDepartment of Chemistry and Institute for Cellular and Molecular Photobiology, University of Nebraska, Lincoln, NE 68588, USA

Stentor coeruleus, a blue-green protozoan ciliate is able to light perception due to cellular pigment, stentorin. An increase in light intensity elicits in a cell a delayed arrest of cell swimming, ciliary reversal followed by restoration of forward swimming in a new direction (step-up photophobic response). Microscope video recording showed that an addition to the medium of a membrane permeable 8-Br-cGMP or IBMX, an inhibitor of cyclic nucleotide phosphodiesterase (PDE), inhibits photosensitivity of *Stentor* to light (the latency of a phobic reaction is longer) as compared to the control. A cytoplasmic cGMP level was determined using radioimmunoassay in dark adapted cells and compared with cells illuminated with different intensity. Light applied evokes a rapid and transient decrease of cGMP level in the cell, followed by the subsequent increase in comparison to the control. Bioelectrical studies revealed that increase of light intensity elicits in *Stentor* a delayed photoreceptor potential followed by an action potential which triggers in turn ciliary reversal. A comparison of changes of measured cGMP level with changes of membrane potential suggests a direct involvement of cytoplasmic cGMP in photosensory transduction pathway in *Stentor* ciliates.

Tu-48 ROLE OF MAMMALIAN MYOSINS I IN THE ENDOCYTIC PATHWAY. (A. Durrbach, K. Collins, P. Matsudaira, D. Louvard, and E. Coudrier) Biologie des Membranes, Institut Pasteur, 25 rue du docteur Roux, 75724 cedex 15 France.

Recent studies have indicated that actin microfilaments participate in membrane trafficking along the regulated exocytosis pathway and the early steps of endocytosis. Myosin Is, a ubiquitous monomeric subclass of myosins exhibiting actin based motor properties and associated with plasma membrane and vesicular membranes, have been suggested as key players for these processes. In order to investigate the functions of myosin Is, we have transfected in a mouse hepatoma cell line (BWTG3), cDNAs encoding the most extensively studied mammalian isoform, the brush border myosin I (BBMI) and 2 truncated variants in the motor domain. One variant lacks the 40 first amino-acids (BBMI-40), the other, the entire motor domain (Tail). The production of each of the two variants impaired the organization of 1) the late endocytic compartment, as characterized by the distribution of internalized transferrin (uptake for 30min), 2) lysosomes characterized by the distribution of cathepsin D and α 2-macroglobulin (internalized for 30min), and 3) the acidic compartment labelled with Acridine Orange. In contrast, the full length BBMI did not modify the structure of these compartments. The number of receptors and the kinetics of internalization and recycling of transferrin and α 2-macroglobulin were similar in control cells and cells producing BBMI or its variants. However, the rate of α 2-macroglobulin degradation was increased by 50% and 35% over control cells, in cells producing BBMI-40 and Tail, respectively. In contrast, the rate was decreased by 20% in cells producing BBMI. When cells were incubated at 18°C, α 2-macroglobulin was blocked in the late endocytic compartment, and its degradation was inhibited in both control cells and cells producing BBMI variants. This indicates that the truncated variants affect the delivery of ligands to lysosomes rather than a missorting of lysosomal enzymes to the early endocytic compartment. Altogether, these data suggest that the BBMI variants may compete with an endogenous myosin I, involved in the organization of the late endocytic compartment and the delivery of ligands to lysosomes.

Tu-49 A CHROMOSOMAL PROTEIN ESSENTIAL FOR CHROMOSOME SEGREGATION AND MITOTIC SPINDLE ASSEMBLY

J.P. Yeo, E. Karsenti*, F. Alderuccio and B.H. Toh
Department of Pathology and Immunology,

Monash Medical School, Melbourne, Australia and

*European Molecular Biology Laboratory, Heidelberg Germany

We have identified an unusual autoantibody which reacts by immunofluorescence only with mitotic chromosomes. The autoantibody immunoblots and immunoprecipitates an evolutionarily conserved 47 kD molecule in interphase and mitotic cells but immunoprecipitates a 47 kD phosphoprotein only from mitotic cells. The 47 kD protein is complexed with 3 other molecules, identified by immunoprecipitation, as 31 kD, 67 kD and 200 kD proteins. The deduced amino acid sequence of the cDNA encoding the 47 kD protein contains 3 consensus motifs for phosphorylation by cdc2 kinase. Phosphoamino acid analysis confirms that the protein is phosphorylated at serine and threonine residues and tryptic phosphopeptide mapping shows that the sites phosphorylated by cdc2 kinase *in vitro* are at the same sites as those phosphorylated *in vivo*. Antisense mRNA knock out or antibody electroporated into cells arrest cells in mitosis at G2/M accompanied by failure of mitotic spindle formation. Accordingly, the molecule has been named RMSA-1 (Regulator of Mitotic Spindle Assembly-1). The 31kD RMSA-1 associated protein is a type 1 phosphatase activated only in mitosis. Activation is mediated by phosphorylation of RMSA-1 by cdc2 kinase. RMSA-1 is proposed to be chromatin factor and regulatory subunit of the phosphatase complex. RMSA-1, phosphorylated by cdc2 kinase at the G2/M transition activates the associated phosphatase and is essential for mitotic spindle assembly and chromosome segregation.

Ref: Yeo, J.P., Alderuccio, F. & Toh, B.H. 1994. Nature 367: 288-291

Tu-51 ACTIN FILAMENTS IN GRAVIPERCEPTION OF THE BASIDIOMYCETE *FLAMMULINA VELUTIPES*

J. Monzer
Lehrstuhl für Botanik, Technische Universität München-Weihenstephan, D-85350 Freising, Germany.

Like plants, basidiomycete fruiting bodies show distinct gravitropic orientation. Elongation of the stipe is regulated to achieve an upright rearrangement of the cap. Cells of the transition zone in the stipe apex are responsible for graviperception in the agaric, *Flammulina velutipes*. As revealed by physiological studies, immunohistochemistry, and video microscopy, actin filaments and the nuclei may be involved in cellular gravity perception in *Flammulina*. Actin filaments are closely co-localized with the nuclei in the stipe hyphae. Explanted stipes show a concentration-dependent, partial inhibition of gravitropic curvature after cytochalasin D treatment. The nuclei of the multinucleate stipe cells show sufficient density and mass for a statolith function. Nuclear motility, mainly present along the longitudinal cell axis, was analyzed for changing frequencies with different spatial orientations of the cell. Cytochalasin treatment causes a decrease also of nuclear motility.

References:

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- Sievers A, Buchen B, Volkmann D, Hejnowicz Z (1991): Role of the cytoskeleton in gravity perception. In: Lloyd CW (ed) The cytoskeletal basis of plant growth and form. Academic Press, London, 103-109.

Tu-50 THE 3-D ARRANGEMENT OF THE PLANT GOLGI APPARATUS IS MEDIATED BY ACTIN

B. Satiat-Jeunemaitre^{a,b}, J. Chan^a and C. Hawes^a

^aSchool of Biological & Molecular Sciences, Oxford Brookes University, Oxford, U.K.; ^bBiomembranes Végétales, C.N.R.S., Université Pierre et Marie Curie, Paris, France.

The relationship between the cytoskeleton, the spatial distribution of Golgi stacks and their BFA induced rearrangement has been studied in maize and onion root cells.

The distribution of the plant Golgi apparatus has been assessed by immunofluorescence microscopy with a monoclonal antibody JIM84, recognising a Golgi glycoprotein¹. After disruption of the microtubule system with colchicine or oryzalin, the staining pattern resulting from numerous cisternal stacks distributed throughout the cytoplasm, was unchanged. However, after depolymerisation of the actin network with cytochalasin the number of fluorescent organelles is greatly reduced along with a concomitant increase in their size. Electron microscopy has shown that this modification is due to a clustering of the Golgi stacks which do however remain intact. This indicates that the spatial arrangement of the Golgi apparatus as a whole, but not the integrity of individual cisternal stacks, is actin mediated.

Our previous work has also shown a redistribution of the JIM 84 epitope with brefeldin A resulting in a massive redistribution of the Golgi stacks and their eventual vesiculation to form "BFA compartments"^{2,3}. Using cytoskeletal disrupting agents we have shown that this BFA induced Golgi disruption is not microtubule mediated.

- (1) Horsley D. et al., 1993. *J. Exp. Bot.* 44, 223-229.
- (2) Satiat-Jeunemaitre B. and Hawes C., 1992. *J. Cell Sci.* 103, 1153.
- (3) Satiat-Jeunemaitre B. and Hawes C., 1993. *Biol. Cell.* 79, 7-15.

Tu-52 EXPERIMENTAL ANALYSIS OF TRANSITION FROM BI- TO TRICELLULARITY IN POLLEN DEVELOPMENT

V. Žářský^a, J. Hašek^b, J. Tupy^a

^a Institute of Experimental Botany, Czech Academy of Sciences, Ke dvoru 15, Praha 6, CZ-160 00, ^b Institute of Microbiology, Czech Academy of Sciences, Videnska 1083, Praha 4, CZ-142 20

Mature pollen of higher plants are arrested before pollination either in the bi- or tricellular stage of development. In bicellular pollen the generative cell division takes place in the pollen tube after germination whereas in tricellular pollen this second pollen mitosis takes place in the anther before pollination. It is thought that species with tricellular pollen evolved from bicellular ones because all taxa considered to be phylogenetically primitive are bicellular. In this report we describe an *in vitro* experimental approach which induces tricellularity in the normally bicellular pollen of tobacco. Changes in pollen cytoskeleton organisation upon transition from bi-to tricellularity will be described.

**TAXOL ACTION ON MICROTUBULES OF CONTROL
AND TAXOL-RESISTANT PLANTS OF *NICOTIANA*
Tu-53 *PLUMBAGINIFOLIA***

A.P. Smertenko, N.M. Strashnyuk, Ya.B. Blume

Inst. of Cell Biology & Genetic Engineering, Acad. of Sci. of the Ukraine, acad. Zabolotnogo str., 148, Kiev DSP-22, 252022, the Ukraine

Previously we obtained mutants of *Nicotiana plumbaginifolia* resistant to 2 μ M taxol (tax2 r) with altered tubulin isoforms (Proc. of XV Int. Botanical Congress, 1993, Tokyo, Japan, p.336). By means of immunofluorescent microscopy with anti-tubulin antibodies Tu-01 (kindly provided by Dr. V. Viklicky, Inst. Mol. Genetics, Prague) it was demonstrated that interphase microtubule (MT) arrays in protoplasts from tax2 r line remain intact after 2-12 h treatment with 2 μ M taxol, while in control cells ones was strongly stabilized. In control regenerating protoplasts taxol increased mitotic index in ~10 times. In these conditions it was possible to identify the different stages of prophase band (PPB). Briefly, broad bands appeared before prophase and narrowed to form a thin well-defined band in prophase. Just prior prophase, MTs which possibly arise from the PPB, collected on the nuclear surface to eventually from the prophase spindle. On this stage abnormal spindle formed (2-4 minispindles could be observed per cell). But when taxol have affected the cell on the earlier metaphase, it dramatically destroyed spindle but minispindles didn't occurred. In both cases highly broken nonfocused spindle poles were characteristic for prophase and metaphase. After 4 h of treatment with 2 μ M taxol all mitotic spindles of tax2 r were bipolar. Hence, the obtained mutant can be used for examination of specificity of spindle pole functioning and polar MT organizers in higher plant cells.

**Tu-55 VINBLASTIN-RESISTANT PLANTS OF *Nicotiana*
plumbaginifolia HAVE A MUTANT β -TUBULIN
WITH DECREASED AFFINITY FOR VINBLASTIN**

V.G. Solodushko, A.I. Yemets, N.M. Strashnyuk, Ya.B. Blume

Institute of Cell Biology & Genetic Engineering, Academy of Sciences of the Ukraine, acad. Zabolotnogo str., 148, Kiev DSP-22, 252022, the Ukraine

Nicotiana plumbaginifolia plants resistant to vinblastine were obtained in order to study the resistance mechanisms of plant microtubules to compounds with antimicrotubular activity. Mesophyll protoplasts and calli were irradiated by γ -rays to induce mutations. The selective concentrations of vinblastine used were in the range of 10-50 μ M. Primary analysis showed the presence of resistance to this alkaloid in regenerated plants. Biochemical analysis of the purified tubulin from several of vinblastine-resistant lines using two-dimensional electrophoresis revealed changes in the mobility of one of β -tubulin isoforms. Further study of tubulin, obtained from these plants, showed its decreased affinity to vinblastine in comparison to tubulin which was obtained from wild *Nicotiana plumbaginifolia* plants. It is supposed, that the changes in given isoform of β -tubulin are a result of the mutation in one of the sites, which is responsible for structural changes in this vinblastine-tubulin binding center.

**CELL DIVISION RESPONSE TO AMIPROPHOS-
Tu-54 METHYL IN AMIPROPHOSMETHYL-RESISTANT
PLANTS AND THEIR SOMATIC HYBRIDS**

L.V. Malysheva, O.P. Kundelchuk, A.I. Yemets, Ya.B. Blume, Yu.Yu. Gleba

Institute of Cell Biology & Genetic Engineering, Acad. of Sci. of the Ukraine, Zabolotnogo str., 148, Kiev DSP-22, 252022, the Ukraine

Cell division response to amiprophosmethyl (APM) evaluated by the mitotic index (MI) level was investigated in root tips of *Nicotiana plumbaginifolia* mutant resistant to 5 μ M of APM (Sapm r), and in two asymmetric somatic hybrids of this plant with *Atropa belladonna*, and *N. sylvestris*. Both hybrids had only one chromosome from Sapm r . In the first hybrid high level of asymmetry resulted from spontaneous elimination of *N. plumbaginifolia* chromosomes after symmetric hybridization, in the second case - it was induced by γ -irradiation of *N. plumbaginifolia* protoplasts before fusion. In all plants, both resistant and sensitive, a pronounced increase in the percentage of metaphases +anaphases among dividing cells was observed after treatment with APM. The value of MI showed strong correlation to herbicide resistance. The mutant Sapm r plants were significantly less sensitive, as compared to the control *N. plumbaginifolia*. After treatments with respective APM concentrations the increase of MI was 6-fold in mutant plants and 27-fold in control ones. Somatic hybrids had MI values intermediate to the parents; they were more resistant than *A. belladonna* or *N. sylvestris*, but more sensitive than Sapm r mutant. Since three β -tubulin genes were localized on different chromosomes of *N. plumbaginifolia*, these results could be interpreted as a consequence of mutation on one of the β -tubulin genes only in Sapm r plants.

**MICROTUBULE-TRANSLOCATING PROTEINS FROM
ISOLATED PHRAGMOPLASTS**

Tu-56
H. Shibaoka and T. Asada
Department of Biology, Faculty of Science,
Osaka University, Toyonaka, 560 Japan

Our previous study suggested the involvement of microtubule(MT)-associated motility systems in the organization of the phragmoplast (Asada et al., 1991). As a part of our efforts to understand the molecular basis of MT-associated motility that is involved in the organization of the phragmoplast, an attempt was made to identify proteins with the ability to translocate MTs in an extract from phragmoplasts isolated from tobacco BY-2 cells.

Homogenization of isolated phragmoplasts in a solution that contained MgATP, MgGTP and a high concentration of NaCl resulted in the release from phragmoplasts of factors with ATPase- and GTPase-activity that were stimulated by MTs assembled in vitro from brain tubulin. A protein fraction with MT-dependent ATPase and GTPase-activity caused minus-end-headed gliding of MTs in the presence of ATP or GTP. Factors with the MT-translocating activity cosedimented with MTs assembled in vitro from brain tubulin and were dissociated from sedimented MTs by an addition of ATP or GTP. After cosedimentation and dissociation procedures, a 125-kDa polypeptide and a 120-kDa polypeptide were recovered in a fraction that supported minus-end-headed gliding of MTs. This fraction contained some MT-associated polypeptides in addition to the 125-kDa and 120-kDa polypeptides, but a fraction that contained only these additional polypeptides did not cause any translocation of MTs. Thus, it appeared that the 125-kDa and 120-kDa polypeptides were responsible for translocation of MTs. These polypeptides with plus-end-directed motor activity may play an important role in the organization of the phragmoplast.

Tu-57 PLANAR PERINUCLEAR BAND OF MTs AND ANASTRAL MEIOTIC METAKINESIS - NORMAL AND ANOMALOUS

N.V.Shamina

Institute of Cytology & Genetics, Novosibirsk
630090, Russia

A highly organized planar meridional perinuclear band of MTs without any prominences or points of convergence has been found in the PMCs of cereals at diakinesis. From NE breakdown on, the band rearranges itself into the system of fibers of central spindle, going through the following steps in succession: 1) a fragmentation of the band into straight bundles of MTs to form a chaotic net; 2) autonomous rotation of the MT bundles for these to gain a roughly bipolar orientation; 3) their parallel coorientation and the formation of an integrated spindle body; 4) convergence on the poles. Fusomes are then localized only within the polar regions of cells. If step 2 is blocked, the formation of polyarchal spindles are observed to form at anomalous meiosis of some cells of distant hybrids; if step 3 is blocked, bipolar disintegrated spindles form, as was observed for the mei10 mutants of rye; if steps 2 and 4 are blocked at a time, there is a unique anomaly seen, namely monoastral plant spindles (as in dv mutants under altered environmental conditions). Metakinesis of the classical dv is noted for all the steps above listed, except for step 4. The formation of only conic bipolar spindles at some types of asynaptic meiosis, as all univalents are oriented in an unipolar way, implies that cMT cannot converge on the poles in the absence of kMTs at meiotic metakinesis. Spindle visualization was done after Navashin.

Tu-59 SEQUENCE CHARACTERIZATION OF ROOT TISSUE-SPECIFIC MYOSIN HEAVY CHAIN ISOFORMS FROM SUNFLOWER, *Helianthus annuus*

Oliver Vugrek and Diedrik Menzel

Max-Planck-Institut für Zellbiologie, Rosenthal, D-68526
Ladenburg, Germany

Twelve positive clones were retrieved from a root-specific sun-flower cDNA library using PCR-generated probes of myosin heavy chain sequences. Five of these were unique and contained complete reading frames. Sequence analysis shows that all five clones are genuine MHC isoforms, but overall homologies were rather heterogeneous ranging from 45% to 81%. Preliminary sequence comparisons with *Arabidopsis*, *Anemone*, and *Acetabularia* MHC sequences suggests that the currently known variants of plant MHCs fall into separate classes, which are recognizable across species borders. This reflects phylogenetic variation as well as functional differentiation in plant myosin heavy chains. It is, therefore, likely that the members of each class in the emerging MHC multigene family serve specialized functions.

Tu-58

ACTO-MYOSIN-BASED DYNAMICS OF ENDOPLASMIC RETICULUM AND CHLOROPLASTS IN VALLISNERIA MESOPHYLL CELLS

Susanne Liebe and Diedrik Menzel

Max-Planck-Institut für Zellbiologie, Rosenthal, D-68526
Ladenburg, Germany

It is well known that actin-based rearrangement of chloroplasts and rotational streaming can be induced in Vallisneria mesophyll cells. By vital staining with the lipophilic fluorochrome DiOC₆, we show here that the rearrangement in chloroplast distribution coincides with a change in the morphology of the endoplasmic reticulum (ER). During induction of streaming, chloroplasts are trapped within the ER network and the behavior of both organelles appears to be mutually dependent. This situation also holds as the rate of streaming increases and tubular ER strands form in the subcortex. These strands remain in close contact with rows of chloroplasts and the entire complex is engaged in rapid movement. Indirect immunofluorescence using an anti-fast-myosin antibody suggests that myosin is not localized on the actin fibres but on basically all membranous compartments, namely mitochondria, ER and chloroplasts. This supports the hypothesis that cytoplasmic streaming is the result of both single organelle movements and mass movements mediated by interactions of the ER network with other organelles as well as with the underlying actin cytoskeleton.

Tu-60 SYMMETRIC HYBRIDS USING β -TUBULIN MUTANTS OF *Nicotiana plumbaginifolia*

A. Yemets, V. Rudas, N. Strashnyuk, Ya.B. Blume

Institute of Cell Biology & Genetic Engineering, Acad. of Sciences of the Ukraine, acad. Zabolotnogo str., 148, Kiev DSP-22, 252022, the Ukraine

Somatic hybridization of higher plants involves reorganization of microtubule systems of parental cells. For studying peculiarities of cytoskeletal functions in hybrid cells nuclear β -tubulin mutants of *Nicotiana plumbaginifolia* resistant to antimicrotubule compounds - amiprofosmetyl (APM) and trifluralin (TFL) - were used as donors. These mutant lines previously obtained by us are mutants with changed isoforms of β -tubulin. To produce symmetric somatic hybrids mesophyll protoplasts of *N. sylvestris* and *Atropa belladonna*, both resistant to kanamycin (Km), were used as recipients. After appropriate selection hybrid clones of *N. plumbaginifolia+N. sylvestris* and *N. plumbaginifolia+A. belladonna* with resistance to Km and APM or TFL were obtained. The cytogenetic analysis justified hybrid origin of the obtained hybrid clones. They have various phenotypes: some of them - of *N. plumbaginifolia*, or *N. sylvestris*, or *A. belladonna*; others - intermediate phenotypes. By immunofluorescence microscopy there was demonstrated that protoplasts of mutant lines and of the obtained hybrid clones conserved the normal structure of interphase microtubule arrays after treatment with APM (5×10^{-6} M) and TFL ($1,2 \times 10^{-5}$ M), whereas protoplasts of the recipient lines were destroyed under these conditions.

Tu-61 INFLUENCE OF MICROTUBULE DISRUPTING AGENTS ON GRAVITROPISM OF TIP-GROWING MOSS PROTONEMA CELLS

Ch. Chaban^a, A. Smertenko^b, R. Ripetskyj^a, Ya.B. Blume^b

^a Institute of Ecology of the Carpathians, Academy of Sciences of the Ukraine, Chajkovskogo str., 17, Lviv; 290000; ^b Institute of Cell Biology & Genetic Engineering, Academy of Sciences of the Ukraine, acad. Zabolotnogo str., 148, Kiev DSP-22, 252022, the Ukraine

Immunofluorescence of dark-grown negatively gravitropic apical cells of the protonema of *Pottia intermedia* showed microtubules (MT) distributed throughout the cytoplasm mainly in axial orientation extending from the plastid free apical zone to the most distal region. Depolymerizing action of oryzalin and amiprotophosmethyl spread from cell apex towards nucleus being directed firstly to thinnest MTs. The angles of negatively gravitropic curve of apical cells, growing on the medium with herbicides were notable for abnormally high variability. On the medium with low herbicide concentrations ($>1 \mu\text{M}$ oryzalin or $1 \mu\text{M}$ amiprotophosmethyl), the cells were able to restore the normal organization of MT system. The typical morphological effect induced by these antimicrotubular agents was an occurrence of the new growing points, some of outgrowths began to grow indicating dynamic stability of MT system. But about 12% of apical cells, which renewed normal growth, did not react adequately to gravistimulation. The results obtained suggest that cortical microtubules system participates in the realization of gravitropic reaction of the protonema cells.

Tu-63 PHOSPHORYLATED PROTEINS RECOGNIZED WITH MPM-2 ANTIBODY-STUDY OF THEIR LOCALIZATION WITH MICROTUBULE ORGANIZING CENTRES OF HIGHER PLANTS

P.Binarová^a, L.Fowke^b, P.Rennie^b

^a Institute of Experimental Botany, Sokolovská 6, 772 00 Olomouc, Czech Republic; ^b Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Set of mitotic phosphoproteins, localized mainly with discrete sites of microtubule organization as are centrosomes or spindle pole bodies is recognized with MPM-2 monoclonal antibody in many eukaryotes. We studied localization of MPM-2 recognized proteins in higher plant cells where the presence of discrete sites for microtubule organization have not been proved yet. We showed that MPM-2 did not localize with most of putative microtubule organizing centres in cells and protoplasts of gymnosperm white spruce (*Picea glauca*) and angiosperm (*Vicia faba*). Immunofluorescence and immunogold microscopy showed that MPM-2 recognized phosphopeptide(s) are localized with centromeres and kinetochores similarly as it is known from mitotic animal cells. MPM-2 recognized phosphoprotein associated with nuclei and chromosomes were studied using electrophoretic techniques. Our results suggest that cell cycle dependent phosphorylation/dephosphorylation of these regions is involved in mitotic events such as chromosome organization and chromatid separation.

Tu-62 CYTOLOGICAL INVESTIGATION OF THE FIRST EVENTS DURING INDUCTION OF MICROSPORE EMBRYOGENESIS IN BARLEY AND WHEAT

P.Binarová, L.Ohnoutková

Institute of Experimental Botany, Sokolovská, 772 00 Olomouc, Czech Republic

Dihaploid plants of barley and wheat with high responsibility to induction of pollen embryogenesis were used.

After cold pretreatment anthers were cultured on medium MN 6(barley) and C 17(wheat) to induce embryogenesis. Cytoskeleton rearrangement and DNA synthesis were studied using immunocytochemical methods during cold pretreatment and the first 4 weeks of culture.

Tu-64 ASSOCIATION OF P34CDC2 KINASE WITH THE PREPROPHASE BAND IN MAIZE CELLS

S.M. Wick^a, J. Colasanti^b, S.-O. Cho^a and V. Sundaresan^b

^a Department of Plant Biology, University of Minnesota, St. Paul, MN, U.S.A.; ^b Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

We have performed double immunofluorescence localization of the maize cell division regulatory kinase p34^{cdc2} and microtubules in meristematic root tip cells and the cells undergoing the formative divisions of the leaf stomatal complex. These studies reveal that the only microtubule array with which p34^{cdc2} associates is the preprophase band (PPB), which predicts the site of the upcoming division, and only at the later stages of PPB development. Disappearance of both the PPB and its associated band of p34^{cdc2} kinase after exposure to oryzalin suggests that proper formation of the microtubule band is necessary for localization of p34^{cdc2} at the division site. Whereas p34^{cdc2} kinase does not appear to be involved in determination of the division site (as the PPB begins its development before the first evidence of localized p34^{cdc2}), it is possible that p34^{cdc2} is involved in fixing the division site, for instance, by molecularly marking the cell cortex along the division site so that cell plate fusion with this site at cytokinesis can be properly achieved.

MICROTUBULES ARE NOT INVOLVED IN LOCAL CELL WALL DEPOSITION IN CHARACEAN INTERNODAL CELLS

Tu-65

Ilse Foissner

Institut für Pflanzenphysiologie,
Universität Salzburg, Hellbrunnerstraße 34,
A-5020 Salzburg, Austria

The arrangement of cortical microtubules was studied during and after formation of cellulose wall appositions (plugs), induced by treatment with 5×10^{-7} M CaCl₂, in internodal cells of *Nitella flexilis*. Although microtubule orientation patterns are different in young and matured internodes plug formation appears identical at both stages. Microtubule depolymerization with oryzalin before and during plug formation has no effect on size, number and morphology of plugs. Microtubules disassemble prior to plug formation and reassembly starts only after cessation of plug growth. Reassembled microtubules are randomly oriented, irrespective of microtubule orientation outside the plug area. The results indicate that microtubules are neither involved in vesicle transport nor in the shaping of plugs.

CYTOSKELETON COMPONENTS OF ISOLATED PLANT PLASMA MEMBRANE VESICLES

Tu-66

A. Sonesson and S. Widell

Dept. of Plant Biology, Section of Plant Physiology,
P.O.Box 7007, S-220 07 Lund, Sweden

We have shown that actin is specifically attached to the inside of isolated plant plasma membrane (PM) vesicles¹. Actin was found in both inside-out and right-side-out PM fractions and could also be recovered, after Triton X-100 extraction of the PM vesicles, in a detergent insoluble fraction.

We are now working on a further characterization of this PM-bound actin and also on how it is attached to the PM.

¹Sonesson and Widell (1993) *Protoplasma* 177:45-52

PROBING NICOTIANA TABACUM MICROTUBULES WITH MONOCLONAL ANTI-TUBULIN ANTIBODIES

Tu-67

A. Smertenko^{a,b}, Z. Opatrný^c, Y. Blume^b, V. Viklický^a and P. Dráber^a

^aInstitute of Molecular Genetics, Prague, Czech Republic; ^bInstitute of Cell Biology and Genetic Engineering, Kiev, Ukraine; ^cInstitute of Crop Production, Prague, Czech Republic

Exposure of tubulin epitopes on microtubular structures of *Nicotiana tabacum* cells (strain VBI, suspension culture) was studied with a panel of 11 monoclonal antibodies recognizing different epitopes on structural domains of tubulin subunits and with an antibody GT335 against glutamylated form of tubulin (A.Wolff et al., Eur. J. Cell Biol. 59:425-432, 1992). Specificity of antibodies was confirmed by immunoblotting and the antibodies were tested on fixed cells and detergent-extracted models. All antibodies against tubulin domains reacted on fixed cells with cortical and cytoplasmic microtubules, mitotic spindles, preprophase bands and fragmoplasts. Differential staining was however observed in unfixed detergent extracted cells. While antibodies reacting with epitopes on C-terminal structural domains stained cytoplasmic microtubules, antibodies against N-terminal structural domains did not decorate any microtubules. The substantial parts of N-terminal structural domains are thus either not exposed on the surface of cytoplasmic microtubules or are masked with interacting proteins. Orientation of tubulin subunits in the wall of plant cytoplasmic microtubules resembles that described for animal microtubules (Dráber et al., J. Cell Sci. 92: 519-528, 1989).

GT335 antibody stained in fixed cells all microtubule structures. Plant tubulins are therefore posttranslationally polyglutamylated and this modification could play role in regulation of interaction of microtubule-associated proteins with plant tubulins. This work was supported by 552407 grant from Czech Academy Grant Agency.

TEMPERATURE AFFECTS DIFFERENTIALLY VARIOUS MT STRUCTURES IN WILD AND COLCHICINE-RESISTANT TOBACCO CELL STRAINS

Z.Opatrný^a, A.P.Smertenko^b

^aResearch Institute of Crop Production, Drnovská 507, 170 00 Prague, CR, ^bInst. Cell Biol.+ Genet. Engineering, Zabolotnovo str. 148, 252022 Kiev, Ukraine

Temperature affects to various extent the morphogenetic response of plant cell and tissue cultures. Generally, cell growth (shape, size), cell division (incidence, orientation) as well as cell differentiation can be pronouncedly modified. To elucidate the mechanism of the effect of extreme temperature (cold stress 10-50°C, heat stress 37°C) on the phenotype of tobacco cell strain VBI-0, the behaviour of microtubular (MT) apparatus was followed by means of immunofluorescent techniques. The temperature responsiveness of both wild (normal) and col^r (selected on the medium with 10⁻³ M colchicine) cell strains was characterized. The preferential sensitivity of some MT-structures (in particular spindle) was confirmed.

Tu-69 TRANSIENT EQUATORIAL RING OF TUBULIN-CONTAINING MATERIAL
DURING NUCLEAR DIVISION OF *Schizosaccharomyces pombe*

Alena Pichová^a and Masayuki Yamamoto^b

^aLaboratory of Cell Reproduction, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic. ^bDepartment of Biophysics and Biochemistry, School of Science, University of Tokyo, Tokyo, Japan

The fission yeast *Schizosaccharomyces pombe* is an excellent model system to study essential features of eukaryotic cell division. Cells grow at both ends by localized cell wall growth and form a septum at the cell equator to accomplish symmetrical cell division. Actin has been shown to be part of the machinery responsible for localized cell wall growth.¹ The possible role of microtubules in shape and cell division control is less evident. Here we report new details of the microtubule organization in *Schizosaccharomyces pombe* not described in previous papers. Our results show new microtubular arrays in cell cycle-related locations. We bring the first evidence of the presence of a transient equatorial ring of tubulin-containing material during nuclear division that may cooperate with the F-actin ring to control the plane of cell division.

1. Drubin, D.G. - Cell Motility and Cytoskeleton, 15, p 7

Tu-70 CORTICAL TUBULIN-CONTAINING STRUCTURES IN SACCHAROMYCES CEREVISIAE

J. Hašek, P. Vavřičková

Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

The reactivity of the mouse monoclonal antibody Tu-14 raised against porcine brain β -tubulin (Dráber et al., J. Cell Sci. 92, 519, 1989) was studied in cells and spheroplasts of budding yeast *Saccharomyces cerevisiae* by means of immunoblotting and immunofluorescence. As compared with the reaction of the polyclonal antibody 206-1 specific for yeast β -tubulin (Bond et al., Cell 44, 461, 1986), a prominent reaction of the antibody Tu-14 in immunoblotting was observed at the position of tubulin. A modified procedure was used for the preparation of cells for the immunofluorescence in order to avoid proteolysis and a sudden rearrangement of the cytoskeletal elements. Besides nuclear and cytoplasmic microtubules, Tu-14 antibody revealed an elaborate network of filaments beneath the plasma membrane in cultivated spheroplasts. The staining pattern of the antibody in cells was similar to the distribution of 210 kD microtubule-interacting protein (Hašek et al., Can. J. Microbiol. 38, 149, 1992). Arrangement of epitopes recognised by Tu-14 antibody was analysed also in *cis* tub2 mutants and cells overexpressing β -tubulin. Results indicate the presence of cortical microtubules in *S. cerevisiae*.

Tu-71 TAXOL REARRANGES NATIVE MICROTUBULE BUNDLES IN A MAP-DEPENDENT FASHION

C. Hunt and R. Stebbings

University of Exeter, Department of Biological Sciences, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, UK.

The anti-mitotic drug, taxol, is known to cause fundamental rearrangements of microtubule arrays within living cells although the mechanism by which it does so is still unclear.

Large parallel arrays of microtubules can be isolated from hemipteran ovaries by virtue of their birefringence and exposed directly to taxol. Within 3 minutes the entire microtubule aggregate coils up into a tight tangle. The reaction differs if the microtubules have been treated with high concentrations of salt in order to strip away any microtubule-associated proteins (MAPs). In this case the aggregates appear straight in outline but with a 'zebra-like' striped appearance when viewed in polarised light which reflects a tight twisting of the microtubule array around a central axis. The microtubule aggregates do not respond to taxol if pre-treated with cationic ferritin which would hinder slippage of adjacent microtubules. This suggests that such sliding plays a role in taxol-induced tangle formation. Coiling is also pH-sensitive. At a pH close to the isoelectric point of tubulin microtubule aggregates fail to react to taxol, while exposure to a high pH inimical to polymerisation causes coiling in the absence of taxol. The similar response of the microtubule array to two treatments which are both likely to weaken interprotofilament bonding [Dye, R.B., Fink, S.P. & Williams, R.C. Jnr. (1993). *J. Biol. Chem.*, 268, 6847-6850] suggests that sliding of adjacent protofilaments, leading to bending of microtubules, might form the molecular basis of this drug-induced reaction.

Tu-72 THE ROLE OF MAP1B IN NEURITOGENESIS

J. Avila, L. Ulloa and J. Díaz-Nido, Centro de Biología Molecular (CSIC-UAM), 28049-Madrid

MAP1B, the first MAP which is expressed in neurons *in situ*, has an essential role in the initiation of neurite growth. This role appears to be regulatory by phosphorylation.

The existence of at least two major modes of MAP1B phosphorylation which can be distinguished by the use of different antibodies to phosphorylation-sensitive epitopes has been recently described. The mode I of MAP1B phosphorylation that might be catalyzed by proline-directed protein kinases, whereas the mode II of MAP1B phosphorylation is catalyzed by casein kinase II.

Interestingly, these two modes of MAP1B phosphorylation are independently regulated during brain development and show a differential subcellular distribution. The mode I of MAP1B phosphorylation strongly diminishes during development in most adult brain regions (Fischer and Romano-Clarke, 1990; Vierck et al., 1989; Ulloa et al., 1993b) (Figure 3). Mode I-phosphorylated MAP1B is localized to the distal growing segments of developing axons and axonal maturation is accompanied by dephosphorylation (Mansfield et al., 1992; Ulloa et al., 1994). No dephosphorylation of mode I-phosphorylated MAP1B occurs in the olfactory system, where there is a persistent growth of axons from sensory neurons of the olfactory epithelium. This supports the view that mode I-phosphorylated MAP1B can be considered as a marker for active axonal growth. The mode II of MAP1B phosphorylation is maintained in the adult brain (Ulloa et al., 1993b) and is present both in axons and dendrites (Ulloa et al., 1994).

Also phosphorylation by casein kinase II at mode II sites on MAP1B may favor its binding to microtubule and may be essential for neurite growth. The functional consequence of the mode I of MAP1B phosphorylation are not yet understood, although it can be speculated that this specific mode of phosphorylation of MAP1B might contribute to the dynamic configuration of microtubules which has been observed in growing axon terminals.

On the other hand it has been found the presence of hyperphosphorylated MAP1B (particularly at mode I sites) associated with dystrophic neurites and neurofibrillary tangles within the brains of patients with Alzheimer's disease, which may be correlated with the aberrant neurite growth, characteristic of this disorder.

Tu-73 DEVELOPMENTAL COMPARISON OF TWO PHOSPHORYLATION EPITOPOS ON MAP1B
M.S. Bush and P.R. Gordon-Weeks,
Developmental Biology Research Centre, The
Randall Institute, 26-29 Drury Lane, WC2B 5RL
Differential phosphorylation of MAP1B generates biochemical and functional diversity. A phosphorylated site recognised by monoclonal antibody (mAb) 150 is associated with axonogenesis, but the relationship between it and another phosphorylation epitope recognised by mAb SMI-31 is unknown. These epitopes on MAP1B were compared in the developing rat spinal cord and in cultured embryonic dorsal root ganglia. In sections of embryonic spinal cord and in cultured neurons, staining with both mAbs initially appears in growing axons, but only mAb 150 stained axons with a gradient. Immunofluorescence of detergent extracted cultures suggested that MAP1B possessing the mAb 150 epitope was bound to bundled microtubules and is also present as a soluble pool. Post-natally, mAb 150 staining of axons declined due to a down regulation of the epitope on MAP1B, whilst mAb SMI-31 staining progressively increased, because of an up-regulation of the epitope on NF proteins. In the adult, mAb 150 staining re-appeared in the somata and dendrites of motoneurons and a sub-population of the SMI-31 stained myelinated axons, this was due to a cross-reaction with heavy NF proteins. Unlike mAb 150, mAb SMI-31 did not stain commissural interneurons at any of the developmental stages examined. In conclusion, we have demonstrated that mAb 150 cross-reacts with heavy NF proteins and suggest that the phosphorylation epitopes on MAP1B recognised by mAb 150 and SMI-31 are developmentally regulated, but different.

Tu-74 A HIGH MOLECULAR WEIGHT MICROTUBULE-ASSOCIATED PROTEIN IN THE HEMIPTERAN, *ONCOPELTUS FASCIATUS*, IS IMMUNOLOGICALLY RELATED TO THE *DROSOPHILA* 205-KDA MAP.

J. Barnett and H. Stebbings
University of Exeter, Department of Biological Sciences,
Washington Singer Laboratories, Perry Road, Exeter,
EX4 4QG, UK.

Ovaries of *Onkopeltus fasciatus* and other hemipteran insects contain massive aggregates of parallel microtubules and studies have revealed a number of high molecular weight microtubule-associated proteins (MAPs). Previously, a heat-stable 260 kDa MAP was isolated and characterised from *Onkopeltus* and found to be species-specific (Anastasi, A., Hunt, C. and Stebbings, H. 1991 *Cell Motil. Cytoskelet.* 19, 37-48). We have now identified a 200 kDa MAP in *Onkopeltus* ovaries that cross-reacts with a polyclonal antibody raised against *Drosophila melanogaster* 205 kDa MAP* (Goldstein, L.S.B., Laymon, R.A. and McIntosh, J.R. 1992 *Proc. Natl. Acad. Sci. USA* 89, 7693-7697) but shows no cross-reactivity with anti-mammalian neuronal MAP antibodies. Microtubule protein from *Onkopeltus* neuronal tissue shows no cross-reactivity when challenged with any of these antibodies. Surveys of cultured cell lines and lower organisms suggest that MAPs with molecular weights around 200 kDa may be common to most cell types and organisms: the family of MAP-4. Although the *Drosophila* 205 kDa MAP shares properties with MAPs of this type, its elucidated microtubule-binding sequence is dissimilar and defines a distinct class of MAPs. Thus, the *Onkopeltus* insect system contains, amongst others, a MAP that is unlike the mammalian neuronal MAPs but similar to *Drosophila* 205 kDa MAP.

* Kindly provided by L.S.B. Goldstein.

Tu-75 *cis*-ACTING SIGNALS AND *TRANS*-ACTING PROTEINS ARE INVOLVED IN TAU mRNA TARGETING INTO NEURITES OF DIFFERENTIATING NEURONAL CELLS

L. Behar, R. Marks, E. Sadot, J. Barg and J. Ginzburg

Department of Neurobiology, The Weizmann Institute of Science,
76100 Rehovot, Israel

Tau microtubule-associated protein is a neuron-specific protein found primarily in axons and is developmentally regulated. The function of tau is in stabilization of microtubules, which is important in establishing and maintaining neuronal morphology. Axonal localization of tau involves a multistep process which is studied in differentiating primary neuronal culture. The initial step involves sorting and subcellular localization of its encoding mRNA into the proximal portion of the axon. Using the transfection assay into neuronal cells, we have demonstrated that a region of 280 nucleotides, located in the 3'-untranslated region, includes a *cis*-acting sequence, which is involved in tau mRNA targeting. In addition, using ultraviolet cross-linking assay, two RNA-binding proteins of 43 and 38 kd were identified, that exhibit specific binding to a minimal sequence of 91 nucleotides located within the same region, which is involved in targeting. These proteins are present in cytoplasmic extracts, prepared from neuronal cells, and in isolated microtubule preparations. These results support a novel model in which *cis*-acting signals together with RNA-binding proteins are involved in the targeting of tau mRNA, that ultimately will lead to its axonal localization.

Tu-76 PHOSPHORYLATION AND ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE BY VASOPRESSIN AND PHORBOL ESTER IN HUMAN BLOOD PLATELETS

O. Aharonovitz, Y. Granot

Department of Life Sciences Ben-Gurion University of the Negev,
Beer-Sheva 84120, Israel

Mitogen-activated protein (MAP) kinases comprise a family of 40-45 kDa protein serine/threonine kinases which are activated by tyrosine and threonine phosphorylation in response to extracellular stimuli. Immunoblotting analysis of human blood platelets with anti-MAP kinase antibodies revealed proteins within the M_r range of 42 to 44kDa. Immunoprecipitates of ³²P-radiolabelled proteins from human blood platelets revealed these proteins which contained low levels of radiolabelling in unstimulated cells. Both vasopressin and phorbol ester rapidly increase the phosphorylation of the MAP kinase proteins. The activation of MAP kinase was estimated according to its ability to phosphorylate several substrates including myelin basic protein, epidermal growth factor-receptor peptide and microtubule-associated protein-2. Our results indicate that 1) MAP kinase activation is rapid, reaching maximal activation levels within two minutes and 2) MAP kinase activation is transient, indicating that the activation signal is attenuated by the action of protein phosphatase. This inactivation process presumably does not involve induction of protein phosphatase expression since blood platelets do not contain active machinery for protein synthesis.

DEPHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEINS (MAP1B AND TAU)

Tu-77K. Szűcs^a, L. Ulloa^b, M.D. Ledesma^b, J. Diaz-Nido^b, V. Dombrádi^a, P. Gergely^a, P. Friedrich^c, J. Avila^b

^aDepartment of Medical Chemistry, University School of Medicine, Debrecen, Hungary; ^bCentro de Biología Molecular, Universidad Autónoma de Madrid, Spain; ^cInstitute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary

The growth and maintenance of neuronal processes depend on microtubules which are mainly composed of tubulin and a group of microtubule-associated proteins. Their hyperphosphorylation has been found associated with dystrophic neurites and neurofibrillary tangles within brains of Alzheimer's disease patients. It was supposed that not only protein kinases, but also protein phosphatases were responsible for the unusual high level of incorporated phosphatase.

In this work rat brain microtubule-associated protein MAP1B, and tau protein, prepared post-mortem from brains of Alzheimer's disease patients were treated *in vitro* with Ser/Thr protein phosphatases [protein phosphatase-1 and -2A catalytic subunits, and -2B (calcineurin) holoenzyme]. The dephosphorylation reactions were monitored by immunoblotting with specific antibodies recognizing phosphorylated and phosphorylatable epitopes. Out of the three enzymes tested, protein phosphatase-2A was only effective in the dephosphorylation of all epitopes.

These data support the view that a dysfunction of protein phosphatase 2A may play a role in the pathogenesis of Alzheimer's disease.

Supported by OTKA 1501, 6003, 6305 (Hungary), CICYT, DGICYT (Spain).

Microtubule-associated proteins 1A and 1B: purification and interaction with microtubules and actin *in vitro*.Khalid Islam^a and Barbara Pedrotti^b,

^aLepetit research Center, GERENZANO (Va); ^bDepartment of Biology, University of Milan, MILANO, Italy.

Several high molecular weight microtubule associated proteins (MAPs) termed MAP1A, MAP1B and MAP2 have been shown to bind to microtubules *in vitro* and *in vivo*. Of these the best characterised is MAP2 which has been shown to stabilise microtubules, lower the critical concentration for assembly, and to bind and crosslink actin filaments. Unlike MAP2, which can be easily purified, MAP1A and MAP1B have not been studied in detail owing to lack of suitable protocols for their purification. We have developed two simple chromatographic procedures for the purification of MAP1A and MAP1B with typical yields of 15 mgs MAP1A and 10 mgs MAP1B protein/kg brain tissue. SDS-PAGE analysis and western blotting using monospecific monoclonal antibodies confirmed that both proteins were over 95% pure with little or no cross-contamination. The ability of purified MAP1A and MAP1B to bind to tubulin dimers and taxol-stabilised microtubules was determined using a novel solid phase immunoassay and showed that both proteins bind efficiently to tubulin dimers and taxol-stabilised microtubules. MAP1A and MAP1B promoted efficient nucleation and elongation of purified tubulin in reconstitution experiments, pure tubulin failed to assemble under these conditions, and electron microscopy confirmed the presence of "normal" microtubules. The association rate constants, calculated from pseudo first-order plots, for MAP1A-, MAP1B-, and MAP2-promoted assembly were roughly $40 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $140 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and $20 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ respectively. The interaction of these MAPs with G-actin and microfilaments was also examined using the solid phase immunoassay and showed that while MAP1A and MAP2 bound to both G-actin and microfilaments MAP1B did not exhibit an interaction. Low-shear viscometry and sedimentation assays further demonstrated that MAP1A is able to cause crosslinking and gelation of microfilaments, although the gelation and crosslinking ability was lower when compared with MAP2.

MAP1A and MAP1B exhibit significant homology at the protein level and complementary temporal expression in brain: MAP1B is particularly abundant in neonatal brain and its expression decreases in mature brain while the reverse is true for MAP1A. The observed differences in the interaction of these two proteins with microtubules and actin may therefore play an important role in "plasticity" events in brain.

DISTRIBUTION OF CYTOSKELETAL PROTEINS IN DAMAGED CELLS OF PARAMECIUM

Tu-78

R. Janisch

Department of Biology, Faculty of Medicine, Masaryk University, 662 43 Brno, Czech Republic

Sectioning of protozoan cells into two fragments strongly interferes with cell integrity and evokes cytoskeletal response manifested by changes in some ultrastructures, including actin distribution, of the injured region.

Using indirect immunofluorescence, changes in the distribution of myosin, tropomyosin, spectrin and vimentin were detected within 10 s of merotomy in *Paramecium caudatum* cells. Myosin tropomyosin were found at high concentrations in the injured region at the site of actin accumulation. Control undamaged cells showed these proteins only in certain cortical structures. Spectrin, which in intact cells is in cortical membranes, was observed under the plasma membrane covering the exposed cytoplasm of sectioned cells. Vimentin present in the ridges enclosing oblong cortical kinetids was seen at high levels in the ridges parallel to the long axis.

The high amounts of myosin and tropomyosin in damaged areas obviously had a role in strengthening the cytoplasm gelified by injury. High amounts of spectrin were probably due to numerous submicroscopic membrane vesicles in the cytoplasm of the damaged area. Spectrin could be involved in linking actin filaments to cortex membranes resulting in contraction of wound edges.

"KINKY" or "STRAIGHT": MICROTUBULE FLEXIBILITY IS MODULATED BY HIGH MOLECULAR WEIGHT ASSOCIATED PROTEINS (HMW-MAPS) IN VITRO

F. Cotelli^a, B. Pedrotti^a and K. Islam^b

^aDept. of Biology, University of Milan, Via Celoria 26, 20133 Milano; ^bLepetit Research Center, Via R. Lepetit 34, 20140 Gerenzano (VA); Italy

HMW-MAPS isolated from brain promote microtubule assembly, alter microtubule dynamics and crosslink microtubules with other cytoskeletal structures. Modulation of these activities imply an important role for these proteins in the organization of the neuritic cytoskeleton. MAP1A, MAP1B and MAP2 are encoded by separate genes and are temporally and spatially regulated. MAP2, which can be easily purified, is perhaps the best characterized as regards its interactions with tubulin and other cytoskeletal structures. We have recently developed purification protocols for the isolation of MAP1A and MAP1B which has allowed us to characterize the biochemical interactions of these proteins with tubulin. Reconstitution experiments using pure tubulin and MAP1A, MAP1B, or MAP2 showed that all three proteins induce tubulin polymerisation and promote both nucleation and elongation. Electron microscopy of MAP1A-, MAP1B-, and MAP2-microtubules exhibited interesting differences in microtubules "kinkiness". MAP1A-microtubules were shorter and essentially "straight" while MAP1B- and MAP2-microtubules exhibited "kinkiness", MAP1B-microtubules appear to be the "kinkiest". MAP1B is an early expressed neonatal MAP, important in differentiation events and neuronal "plasticity", while MAP1A, which shows extensive primary sequence homology with MAP1B, is a late MAP expressed largely in mature brain. The observed differences suggest that MAPs can regulate microtubule flexibility and this regulation may be important *in vivo*.

Tu-81

MAP KINASE ACTIVITY DURING THE FIRST MITOTIC CELL CYCLE OF THE SEA URCHIN EMBRYO

R. Phillipova, B. Glidden, R. Patel and M. Whitaker
Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK

Mitogen-activated protein (MAP) kinases (also known as extracellular signal-regulated kinase, ERK) have been shown to be activated by virtually all mitogens (1). These kinases are important intermediates in the signal transduction pathway. Previous studies have indicated that MAP kinase is activated during mitosis (2) and after fertilization of clam oocytes (3).

The aim of this study was to determine whether MAP kinase activity is present during the first mitotic cell cycle following fertilization of sea urchin eggs (*Lytoclinus pictus*).

We have used two different polyclonal antibodies against ERK for immunoprecipitation and Western blotting. Both antibodies reacted with a single 42kD band. Immunoprecipitations followed by kinase assays show 5 to 10 fold stimulation of activity against myelin basic protein (MBP) just before and during mitosis. The ERK antibody also precipitated a polypeptide that cross-reacted with an antibody to the PSTAIR region of cdc2. We have also carried out histone H1 kinase assays on the ERK immunoprecipitates. Comparisons indicate that 70% of the kinase activity is directed against MBP as substrate.

Our preliminary in-gel assays for MAP kinase activity show an activity at 42 kD against MBP increasing just prior to mitosis.

These data suggest that MAP kinase activity increases during mitosis of the first cell cycle in the sea urchin embryo.

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CYTOSKELETON OF MOTILE MYCOPLASMAS

S. Borchsenius

Tu-83
Department of Cell Culture, Institute of Cytology, Russian Academy of Science St.-Petersburg, Russia

Mycoplasmas lack a cell wall and are amongst the smallest prokaryotes. Albeit the apparent simplicity of their organization and extremely small genome sizes, some mycoplasmas possess so-called gliding motility, the molecular mechanisms of which are unknown. Probably inner cytoskeletal elements participate in the organization of non-spheroidal motile cells motility, as in eukaryotic cells.

Recently we revealed a submembrane system of tubular structures in gliding mycoplasma (*Mycoplasma gallisepticum*), which might perform the function of cytoskeleton. A protein with a molecular mass of about 40 kDa, reacting with antibodies to eukaryotic tubulin, was found in contents of the tubular structures of *M. gallisepticum* (1). Besides, in genomes of three mobile mycoplasmas, including *M. gallisepticum*, some fragments homologous to yeast actin gene have been revealed by DNA-DNA hybridization.

We suggest, that tubular system found in *M. gallisepticum* cells participate in cell movement by means of reversible anchoring of surface proteins on tubules and directional transportation of them along the mycoplasma surface membrane. We called such hypothetical type of mycoplasma cell motility as "caterpillar" movement.

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INTERACTION OF MOUSE PROTEIN CONTAINING HIGH MOBILITY GROUP BOX WITH MICROTUBULES
Tu-82

P. Dráber, M. Zíková and Dráberová E.

Institute of Molecular Genetics, Prague, Czech Republic

Using monoclonal antibodies MA-01 and MA-02, recognizing in various cell lines 210 kDa microtubule-interacting protein (Dráberová and Dráber, *J. Cell Sci.* 106: 1263-1273, 1993), we isolated immunoreactive clones from mouse 3T3 λgt11 expression library and sequenced overlapping cDNA fragments. Polyclonal antibody raised against fusion protein reacted in immunoblotting with 210 kDa protein in whole cell 3T3 lysates and in material immunoprecipitated by MA-01 antibody. Affinity purified polyclonal antibody as well as monoclonal antibodies, raised against fusion proteins and reacting with non-identical antigenic determinants, stained in fixed, detergent-extracted cells microtubular structures and vinblastine paracrystals. The predicted amino acid sequence showed that the protein has distinct acid and basic regions and contains high mobility group (HMG) box involved in binding of DNA. Comparison with other sequences in the database revealed that the protein is identical to mouse T160 that binds to recombination signal sequences (Shirakata et al., *Mol. Cell Biol.* 11: 4528-4536, 1991). Binding assays using taxol-stabilized microtubules and various fusion proteins indicated specific binding of fusion proteins containing C-terminal domain of protein to microtubules. The binding, however, was not inhibited by 1 M NaCl as in the case of control MAP-2 proteins. This work was supported by 552407 Czech Academy Grant Agency.

THE N-TERMINAL DOMAIN OF TALIN: INITIAL STRUCTURAL AND FUNCTIONAL STUDIES

Tu-84
R. P. Grant, Z. H. Rao†, D. I. Stuart† and D. J. G. Rees
The Sir William Dunn School of Pathology, and the
†Department of Molecular Biophysics, University of Oxford, South Parks Road, OXFORD, OX1 3RE, United Kingdom.

Talin belongs to the Band 4.1 family of cytoskeletal proteins, which includes Band 4.1, the ezrin/moesin/radixin/merlin family and two protein tyrosine phosphatases. With the exception of the phosphatases, these proteins have been shown to localize to the ends of actin filaments under the cell membrane. Radixin has been shown to have barbed end capping activity for actin polymerization, while talin has been reported to affect the polymerization of actin *in vitro*. The common sequence relationship in the family is in the N-terminal domain, and we postulate that this domain is involved in the regulation of actin polymerization.

In addition, talin is a substrate for calpain II, which cleaves talin between the 50 kDa N-terminal domain and the C-terminal rod-like tail. We have expressed a fusion protein in *E. coli* of maltose binding protein and the N-terminal domain of talin, corresponding to the 50 kDa cleavage product. The protein expressed is the correct size by protein sequencing and mass spectroscopy. Antisera raised against this fragment react with talin on immunoblots and stain focal contacts of permeabilized fibroblasts.

We have used the recombinant protein in initial crystallization studies and obtained diffracting crystals. We are currently exploring conditions to obtain larger crystals suitable for structure determination. These should enable us to solve the 3-dimensional structure of the first member of the band 4.1 family.

We have also studied the role of talin in actin polymerization: The critical concentration for actin polymerization appears to be unaffected by talin under physiological salt concentrations. We are investigating the role of phospholipids and extending these observations with the use of recombinant protein.

Tu-85 IDENTIFICATION OF FUNCTIONAL DOMAINS IN THE CYTOSKELETAL PROTEIN TALIN
V. Niggli^a, S. Kaufmann^b, W.H. Goldmann^b and G. Isenberg^b

^aDept. of Pathology, University of Bern, Switzerland and

^bBiophysics Department, Technical University of Munich, Garching, Germany

The cytoskeletal protein talin potentially plays a key role in actin-membrane linkage. This protein is able to nucleate actin filament growth *in vitro* while binding simultaneously to bilayers. Thrombin treatment of platelet talin yields two domains of 200 and 47 kDa. We have analyzed the functional properties of the purified fragments. Effects on actin polymerization were studied using fluorescence and viscosity assays. Clearly, the 200-kDa fragment promoted actin filament polymerization and reduced viscosity of actin filaments when present during polymerization, comparable to the effects of the intact protein, whereas the 47-kDa fragment was inactive. In contrast, the 47-kDa fragment, but not the 200-kDa fragment, interacted specifically with large liposomes containing acidic phospholipids. Moreover, we could demonstrate selective hydrophobic photolabelling of the 47-kDa fragment using phosphatidylserine liposomes containing trace amounts of a photoactivatable phospholipid analogue. The 200-kDa fragment, whether alone or together with the 47-kDa fragment, did not incorporate significant amounts of label. These data confirm the validity of the labelling experiments. Moreover, we provide evidence for the existence of distinct functional domains in the protein talin which enable it to interact simultaneously with actin filaments and with bilayers.

Tu-87 NEAR-ULTRAVIOLET LASER IRRADIATION EFFECTS ON CELLULAR ADHESION
V.F.Dima, I.N.Mihalescu*, V.Vasiliu* and S.V.Dima.
 Cantacuzino Institute, *Institute of Atomic Physics, Bucharest, Romania.

The interaction between HeLa tumoral cells and human fibroblasts after near-ultraviolet laser irradiation (337.1 nm) have been studied using an *in vitro* cell invasion model.

The results emphasized the following aspects:

- (1) In non-irradiated cell cultures, we noticed three interaction stages : adhesion, tumoral spreading and displacement with fibroblast; destruction : on the other side, we found a reduced adhesion to non-irradiated human fibroblasts of laser irradiated tumoral cells.
- (2) Significant percent increasing of non-irradiated tumoral cells adhesion to fibroblasts monostrate, irradiated with various laser fluencies (e.g. 0.2 kJ/m² -48.1%; 0.8kJ/m² -63.8% and for 1.6kJ/m² -79.5%). This phenomenon evidenced the close interrelation between fluencies and tumoral adhesion rate.
- (3) The importance of numerical ratio between tumoral cells and fibroblasts in tumoral adhesion and invasion processes (e.g. ratio 1:10-54.7%; 1:5-25.9%; 1:1-54.2% and for 2:1-83.9%) after exposure to high and very high laser fluencies (1.6-6.4kJ/m²).

Summing up, our results emphasized near-ultraviolet laser irradiation effects upon some of tumoral adhesion and invasion mechanism and demonstrate the interrelations between cell populations manifesting different vital potential.

Tu-86 THE LENS-SPECIFIC INTERMEDIATE FILAMENT PROTEINS PHAKININ AND FILENSIN FORM HETEROPOLYMERS IN VITRO AND IN VIVO

A. Merdes*, F. Gounari, F. Schwesinger and S.D. Georgatos. EMBL, Meyerhofstr. 1, D-69012 Heidelberg, Germany; * present address: Dept. of Bio. Chem., Johns Hopkins University, 725 N. Wolfe Street, Baltimore MD 21205, U.S.A.

Phakinin and filensin represent lens-specific proteins of 47 kD and 110 kD respectively. Cloning and sequencing of their cDNAs reveals that phakinin and filensin are new intermediate filament proteins. However, the primary structures of both proteins show several unconventional features: phakinin is a tailless intermediate filament protein, distantly related to type I-keratins. Filensin is a remote member of the intermediate filament protein family with an unusually short rod domain, missing 29 amino acids in coil 2. Both proteins fail to form typical intermediate filaments when individually reconstituted *in vitro*. Nevertheless, a mixture of phakinin and filensin at a molar ratio of 3:1 polymerizes into regular 10 nm-filaments. These filaments have a smooth surface and can also be detected in lens cell homogenates by antibody/protein A-gold labelling.

Tu-88 FREEZE FRACTURE, DEEP ETCH AND FREEZE SUBSTITUTION TECHNIQUES TO STUDY THE CONTRACTILE APPARATUS IN ULTRARAPIDLY FROZEN SMOOTH MUSCLE.
J.L. Hodgkinson, S. B. Marston, T. M. Newman and N. J. Severs. Department of Cardiac Medicine, National Heart and Lung Institute, Dovehouse St., London, SW3 6LY, UK.

The structure of the contractile apparatus in smooth muscle is not fully understood owing to its relative disorder compared to striated muscle. Using quick freeze, deep etch techniques, we eliminate the use of chemical fixatives and skinning agents and can study the ultrastructure in three dimensions. In freeze fracture replicas of ultrarapidly frozen, relaxed, intact *taenia coli*, muscle filaments are well and preserved; the 5.4 nm repeat of the actin sub-units can be seen. In freeze substituted tissue filaments appear more tightly packed than in extracted tissue and less well defined. Thick filaments are difficult to distinguish and are more clearly seen in freeze substituted samples, using a technique specifically tailored to the preservation of smooth muscle. Thick filaments are most easily distinguished from thin filaments in obliquely fractured tissue; here their distribution is comparable to that seen in transverse sections from freeze substituted tissue. Freeze fracture of relaxed, fixed, Triton-extracted tissue (using a method which preserves the contractility of the muscle) reveals the contractile apparatus more clearly. Filaments are more deeply etched; cross-links between filaments, thick filaments and dense bodies are more easily observed. Preliminary data comparing relaxed and rigorized extracted muscle indicate a more ordered structure in rigor; there appear to be more cross-links between filaments, and the face polarity of the thick filaments is clear in freeze substituted tissue. Funded by Wellcome Trust (034508/2/91).

CELL HISTOTYPE-ASSOCIATED GROWTH FEATURES CAN INFLUENCE SUSCEPTIBILITY TO CELL DEATH INDUCED BY TERT-BUTYL-HYDROPEROXIDE

Tu - 89

W. Malorni, G. Rainaldi, R. Rivabene, E. Straface, and M. T. Santini

Department of Ultrastructures, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

The effects of the oxidizing agent tert-butyl-hydroperoxide (t-BHP) were investigated on three human cell lines of different origin and growth features (A431 epithelial cells, ADF astrocytoma cells and U937 leukemic cells) by using immunofluorescence and electron microscopy, and electron paramagnetic resonance spectroscopy (EPR). The results indicate that important biophysical and ultrastructural modifications are induced in the plasma and mitochondrial membranes of these cells and that these changes ultimately can lead to cell death. In particular: 1) an evaluation of membrane order (an indicator of membrane "fluidity") detected by EPR showed a significant decrease in t-BHP treated cells, perhaps as a consequence of stress-dependent lipid peroxidation; 2) a study regarding mitochondrial function conducted by immunofluorescence analysis, using the fluorescent dye Rhodamine 123, showed strong alterations in transport of this dye in cells treated with t-BHP. In order to obtain information about the redox-state of cells, an analysis of intracellular GSH levels was conducted. A marked depletion of this enzyme resulted after t-BHP exposure in all cell types examined. In addition, the cell cytoskeleton also appears to be a target of hydroperoxide-mediated stress. In fact, all three cell types undergo cytoskeletal alterations leading to surface blebbing, a typical characteristic of cell damage, after treatment with this hydroperoxide. However, the timing and extent of this damage as well as that occurring at the mitochondrial and plasma membrane levels seems to be different: cells with weak (ADF) or absent (U937) cell-to-cell and cell-substrate contacts and a poorly developed cytoskeleton appear to be more susceptible than other cell types (A431) to t-BHP-mediated injury.

Tu - 91

Mapping structure-function relationships in human profilin

Björkegren C., Lindberg U. and Karlsson R.
Department of Zoological Cellbiology, E5, W-GI,
Stockholm University, S-106 91 Stockholm,
Sweden.

Profilin is an ubiquitous actin-binding protein regulating the actin polymerization linked to tyrosine kinase-receptor mediated signal transduction in mammalian cells.

In addition to actin, profilin binds to poly(L-proline) and phosphatidylinositol-(4,5)-bisphosphate. Recently the structure of the profilin-actin complex was solved to 2.5 Å resolution (Schutt et al., *Nature* 365:810-816, 1993), and the poly(L-proline) binding site of profilin was identified by site-specific mutagenesis (Björkegren et al., *FEBS Lett.* 333:123-126, 1993) and by NMR (Sharon et al., *FEBS Lett.* 337:145-151, 1993). These studies revealed similarities of profilin to src-homology 3 domains (SH3-domains) known to mediate protein-protein interactions by binding to proline-rich stretches in their target proteins. This further emphasizes the role of profilin in linking the dynamics of the microfilament system to transmembrane signalling.

We are using site-specific mutagenesis of actin and profilin to study structure-function relationships in these proteins, particularly with respect to the dynamics of the microfilament system (for instance; Aspenström et al., *FEBS Lett.* 323:163-170, 1993).

Cyclophilins from *Trichomonas vaginalis* and their implication for cyclosporin toxicity

Tu - 90

K.Heinrich¹, A.Marinets¹, E.Gaugusch¹, H.Erben², P.Johnson³, J.Kulda⁴, J.Graf¹, G.Wiedermann², O.Scheiner¹, M.Duchêne^{1,2} and T.Thalhammer¹.

¹Dept. General & Exptl. Pathology, ²Dept. Spec. Prophylaxis & Tropical Medicine, University of Vienna, ³Dept. Microbiology & Immunology, University of Los Angeles, California, USA, ⁴Dept. Parasitology, Charles University, Prague, Czech Rep.

Trichomoniasis caused by the microaerophilic protozoan *Trichomonas vaginalis* is a highly prevalent sexually transmitted disease. Its treatment is effective with metronidazol, but about 8% of cases of trichomonal vaginitis are refractory to standard treatment. Therefore new methodes to eliminate the parasite would be desirable.

The aim of the study was to investigate if cyclophilin could be the target for antiproliferative drugs, like cyclosporin A (CsA) or its non-immunosuppressive derivatives.

In vitro assays under microaerobic conditions revealed a minimum inhibitory concentration (MIC) of <2ug/ml CsA (cyclosporin A) after 48h to inhibit growth, while the minimal lethal concentration was >10ug/ml. To isolate and characterize the binding proteins for cyclosporins we separated cytoplasmic proteins of homogenized *Trichomonas vaginalis* by DEAE-Sephadex CL-6B chromatography and subsequently a 17kDa protein was eluted by CsA from a CsA affinity-column.

Future studies will examine the effect of cyclosporins on the enzymatic activity and expression of cyclophilins as well as their physiological function in *Trichomonas vaginalis*.

Supported by grant 214/92 of the "Anton-Dreher Gedächtnissstiftung", Vienna.

BIOCHEMICAL CHARACTERIZATION OF CHICKEN CALCYCLIN - SIMILARITIES TO MAMMALIAN CALCYCLIN

A. Filipek, U. Wojda, J. Kuźnicki

Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warsaw, Poland

Calcyclin is a calcium binding protein with two EF-hand domains. For the first time calcyclin protein was purified from mouse Ehrlich ascites tumor cells (Kuźnicki and Filipek, 1987), but so far it was isolated also from rabbit lung and human platelets. In a calcium dependent manner, mammalian calcyclin interacts with annexin II, annexin VI, annexin XI and glyceraldehyde-3-phosphate dehydrogenase. Some suggestions have been made that calcyclin may be involved in cell cycle progression, cell differentiation and secretion. To learn more about possible calcyclin function we analyzed calcyclin which was purified from chicken gizzard by the method developed to purify calcyclin from mouse EAT cells. Chicken calcyclin bound to phenyl-Sepharose in a calcium dependent manner, bound ⁴⁵Ca on nitrocellulose filters and interacted with annexin II, in a manner similar to mammalian calcyclin. The question arises what is a cell specific distribution of chicken calcyclin in smooth muscle, since mammalian calcyclin was found at high level in fibroblasts and epithelial cells of different tissues examined. Further studies will be undertaken to answer this questions and to learn more about calcyclin functions in the cell.

This work was supported by grant from KBN to Dr. Anna Filipek and statutable to the Nencki Institute.

Tu-93 DETECTION OF VIMENTIN IN RELATION TO HUMAN SPERM STRUCTURAL ABNORMALITIES

Ts. Marinova*, R. Fichorova*, R. Stanislavov^b, V. Vickicky^c

^aDept. of Biology and ^bSeminologic Laboratory of Medical Faculty, Sofia; ^cInstitute of Molecular Genetics, Cz. Ac. Sci., Prague

Recent data have shown that specific distribution of intermediate filament proteins in human spermatozoa may correlate with distinct abnormalities of sperm functions. The present study was focused on detection of vimentin by monoclonal antibodies in sperm from normozoospermic and asthenozoospermic ejaculates. Indirect immunofluorescence and immunogold electron microscopy were applied.

Vimentin was always detected in the equatorial segment and postacrosomal sheath. The ultrastructural data revealed additionally intensive vimentin binding in the acosome, neck and initial segment of the middle piece, only in sperm with morphological aberrations: large cytoplasmic droplets, excessive membrane formations in the head, nuclear vacuoles with membranous inclusions, mitochondrial disassembly. These abnormalities were often associated with the presence of polarized colloidal gold granules in the subplasmaemmal and subacrosomal spaces.

Our results suggest that the vimentin may be used as positive marker of specific sperm structural defects with impact in male infertility.

Tu-95 6-DMAP, AN INHIBITOR OF PROTEIN KINASES, BLOCKS PRONUCLEAR FUSION AND MACRONUCLEAR DEVELOPMENT DURING CONJUGATION OF *Tetrahymena thermophila*

Włoga D., Kaczanowski A., & Kaczanowska J.

Department of Cytophysiology, Warsaw University, Warsaw 00-927/1, Poland.

The process of conjugation in a ciliate *Tetrahymena thermophila* results in differentiation of the new macronuclei and resorption of the old one. The micronucleus undergoes meiosis and then one of the meiotic products undergoes premeiotic mitosis yielding in pronuclei; one of them (the migratory one) being attached to the conjugation cell junction in each stage. After cross fertilization and pronuclear fusion, the synkaryon undergoes two postmeiotic divisions. Their products differentiate into macronuclear anlagen and micronuclei. It was known, that beginning from the stage of pronuclei, the nuclei are already committed to the macronuclear development even if their fusion and postzygotic divisions are blocked. This commitment is correlated with appearance of perinuclear "basket" at the stage of pronuclei (microtubules and 14 nm 49 KD microfilaments).

The inhibitor of phosphorylation, 6-DMAP arrested the postzygotic development i.e. the pronuclei did not fuse, did not divide and they did not develop into macronuclear anlagen, while the old macronucleus was not resorbed. It is likely that 6-DMAP inhibited process of phosphorylation of one of the components of the perinuclear "basket" surrounding pronuclei. The developmental window corresponds to the time of premeiotic mitosis and of the stage of pronuclei. Similar developmental block was observed during conjugation of mutant cells bcdxpcd. These conjugants could be rescued with a glucose treatment inducing them into a postzygotic pathway [1]. Similarly, in this study, concomitant treatment of the wl conjugants with 6-DMAP and glucose abolished the developmental block, which was induced by the drug in the sugar-free medium.

[1] Cole E. 1991 Conjugational blocks in *Tetrahymena* pattern mutants and their cytoplasmic rescue. Dev. Biol. 148: 403-419

Tu-94

NON-COVALENT DIMERS OF CALCYCLIN AND ITS CNBr-FRAGMENTS

U. Wojda, A. Filipiak, J. Kuźnicki

Nencki Institute of Experimental Biology, 3 Pasteur str., 02-093 Warsaw, Poland

Calcyclin is a calcium binding protein belonging to the S-100 family. Calcyclin interacts with annexin II, annexin VI, annexin XI and glyceraldehyde 3-phosphate dehydrogenase in a calcium dependent manner in vitro. Since many S-100 proteins form dimers which in contrast to monomers exhibit biological activities (for instance they bind targets), we undertaken studies on dimerization of mouse calcyclin. The S-S dimers were separated from -SH (reduced) forms of calcyclin using chromatography on organomercurial agarose (OMA). It was found using crosslinker that calcyclin eluted with DTT from OMA was able to form dimers by non-covalent bonds. Moreover, in fresh cytosol from mouse Ehrlich ascites tumor cells the S-S dimers were not observed and the main pool of calcyclin seemed to exist as a non-covalent dimer. Similarly, rabbit and chicken calcyclin exists mainly in non-covalent dimer form. Further studies using mouse calcyclin CNBr-fragments showed that both fragments (N-terminal fragment containing cysteine residue as well as C-terminal one without any cysteine) were able to dimerize. The strong tendency of calcyclin to dimerize by non-covalent forces, which was not destroyed by CNBr cleavage of the protein, suggests that dimerization may be especially important in regard to calcyclin functions.

This work was supported by grant from EC East-West programme and statutable to the Nencki Institute

Tu-96 CLONING OF DP71, A MAJOR PRODUCT OF DYSTROPHIN GENE IN THE NERVOUS SYSTEM.

M. Ceccarini, G. Rizzo, P. Macioce, T.C. Petrucci
Laboratory of Cell Biology, Istituto Superiore di Sanità, Roma - ITALY

Duchenne muscular dystrophy (DMD) is a fatal X-linked inherited disease characterized by a defect in dystrophin, a cytoskeletal-plasmaemmal linker protein. Cognitive impairment is a common feature of DMD but underlying mechanisms are still unknown. Dystrophin exhibits complex transcriptional regulation in brain and has a putative role in organizing membrane proteins within neuronal somadendritic microdomains. Two products of the dystrophin gene are expressed in brain: a 427 Kd protein and a 71 Kd protein (Dp71). The latter seems to be the major product of the DMD gene in the nervous system. The identification of the neuronal components that interact with the Dp71 could give clues for elucidating its function. For this purpose, we have cloned the Dp71 by RT-PCR from human fetal nervous tissue and subcloned the sequence in a prokaryotic expression vector. Production of a polyclonal specific antibody is being obtained through rabbit injection with the recombinant protein. Binding experiments with the recombinant Dp71 are in progress to identify the interacting elements in the nervous tissue.

**SUBCELLULAR DISTRIBUTION OF TAU
Tu-97 POLYPEPTIDES**

D. C. Cross and R. B. Maccioni

Lab. Cell. Mol. Biology, Faculty of Sciences, Univ.
of Chile & Int. Ctr. for Cancer and Developmental
Biology (ICC), Casilla 70111 Santiago 7, CHILE.

Our research interest is to investigate *in vivo* functions of Tau proteins. The studies indicate that Tau is a widespread family of polypeptides. Several Tau components were isolated from different non-neuronal sources. Within the context of an analysis of subcellular localization of Tau isoforms in various cell types, we developed polyclonal antibodies to brain Tau and site-directed antibodies against peptides bearing specific sequences of Tau isoforms. Immunofluorescence studies indicated that Tau-like components co-localize with microtubules and stress fibers. It was noteworthy to observe co-localization of immunoreactive Tau's with tubulin in the centrosomes, using a polyclonal antibody against whole Tau. Antibodies to a peptide bearing the sequence of the first imperfect repeat of mature brain Tau did not stain centrosomes, possibly because this Tau sequence is involved in tubulin interactions. On the other hand, mitotic chromosomes were also stained with antibodies against whole Tau and with site-specific antibodies raised against a peptide bearing the N-terminal Tau sequence, in agreement with findings on the presence of nuclear Tau components. Immunostaining was continuously distributed over the chromosomal structure. These findings suggest that Tau polypeptides may play a major structural role in mediating interactions in both interphase and mitotic cells. (Supported by The Council for Tobacco Research, Fondecyt and DTI-Universidad de Chile).

**ACQUISITION OF A POLARIZED PHENOTYPE BY
Tu-99 A MONOCHROMOSOMAL HEPATOMA HYBRID**

C. Delagebeaudeuf, C. Decaens, T. Bedda and D. Cassio
URA 1343 CNRS, Institut Curie, Centre Universitaire,
Bât. 110, Orsay, 91405 Cedex, France

Studies of hepatocyte polarity, an important property of liver cells have been hampered by the lack of valid *in vitro* models. We have previously shown that hepatoma hybrid cell lines constitute a potential reservoir of polarized cells. We have isolated a rat hepatoma-human fibroblast subclone, WIF 12-1, and a derivative WIF-B, that constitute the first model of a well polarized line of hepatic origin. Here we report that the more simple line 11-3, a monochromosomal hybrid obtained by fusion of Fao differentiated rat hepatoma cells with microcells of mouse fibroblast, has acquired a polarized phenotype.

In contrast to Fao parental cells, 11-3 in confluent cultures form bile canaliculi-like structures (BC), that accumulate fluorescein, a property of BC *in vivo*. The distribution of several proteins specific to the hepatocyte membrane poles was studied by indirect immunofluorescence. These proteins were uniformly distributed on Fao cell membrane while the apical markers were concentrated in the 11-3 BC and the basolateral markers excluded from these structures. Moreover the tight junction-associated protein ZO1 was present in belts marking the boundary between 11-3 apical and basolateral poles.

11-3 cells were subcloned: most of the subclones exhibited the polarized phenotype, proving the stability of this line. Two particular subclones were isolated, that formed very few or no BC. Based on the localisation of the ZO1 protein, one subclone appeared non polarized like the Fao parent, and the other showed a polarity similar to intestinal or renal cells with a ZO1 crown at the cell apex. Experiments are in progress to determine if the acquisition of polarity is linked to the single mouse chromosome present in these hybrids.

Compared to our first model WIF 12-1, our second model 11-3 (despite a less pronounced polarity) presents several advantages. 11-3 grows fast, is easy to culture. Its chromosomal content and its expression level of hepatic proteins are close to those of Fao

**INTEGRINS EXPRESSED ON THE APICAL DOMAIN
OF FRT THYROID CELLS POSSIBLY MEDIATE
COLLAGEN-INDUCED REVERSAL OF POLARITY.
Tu-98 C. Garbia^a, R. Negri^a, G. Cali^a, G. Tarone^b and L.
Nitsch^a**

^aC.E.O.S. - Dip. Biologia e Patologia Cellulare e Molecolare, Napoli;
^bDip. Genetica, Biologia e Chimica Medica, Torino, Italy.

It is known that collagen can induce polarity reversal of epithelial cells by interacting with their apical surfaces. We have tried to analyze the early changes induced by this interaction and to identify receptors that may mediate the binding of collagen fibers to the apical cell surface.

FRT thyroid epithelial cells were grown to confluence on filters in bicameral systems. A collagen solution was added to the apical compartment of the chamber. Within two hours after collagen gelification a dramatic drop in the transepithelial resistance was observed. Cells interacting with collagen showed a rearrangement in the distribution of actin and vimentin suggesting that they were undergoing changes in their overall organization. We determined that FRT cells synthesize several integrins, in particular the $\beta 1$ and $\alpha 1$ subunits. By immunofluorescence we showed that $\alpha 1\beta 1$ complexes, which are receptors for the collagen molecules, were predominantly expressed on the basolateral cell surface. However, in a fraction of the cell population, these complexes were detected on a domain of the apical plasmamembrane that appeared to have a peculiar composition since antigens recognized by the HPA lectin were concentrated there. Other basolateral antigens, such as the Na/K ATPase, the 35-40 kD protein, laminin and fibronectin were often expressed on this domain. In samples double stained with antibodies to $\alpha 1$ or $\beta 1$ integrin and type I collagen several examples were observed of collagen fibers interacting with integrin molecules in the apical domain.

We transfected FRT cells with a full length cDNA coding for $\beta 1B$, a variant of the $\beta 1$ integrin that is characterized by an altered sequence of the cytoplasmic domain and is thought to behave as a dominant negative regulator of cell-matrix interaction. Positive clones that have been selected are being tested for their ability to respond to collagen interaction. We hope to be able to gain a direct proof that the $\beta 1A$ integrins are involved in transducing signals generated by the apically bound collagen and responsible for cell polarity reversal.

**Filamin inserts into lipid membranes: A calorimetric, film balance and lipid labeling study.
Tu-100 W.H. Goldmann^a, V. Niggli^b and G. Isenberg^a**

^aDep. of Biophysics E22, Technical University of Munich^bPathology, University of Bern SWITZERL.

The actin-binding protein, filamin interacts with mixtures of zwitterionic and/or anionic phospholipids: dimyristoyl-L-a-phosphatidylcholine (DMPC); dimyristoyl-L-a-phosphatidylglycerol (DMPG); phosphatidylcholine (PC) + phosphatidylserine (PS).

Filamin-lipid interactions of reconstituted lipid bilayers were investigated by (hs) differential scanning calorimetry. These measurements were performed with multilamellar vesicles (MLVs) of $\phi \leq 100$ nm and large unilamellar vesicles by extrusion technique (LUVETs) of $\phi > 100$ nm. By using DMPC MLVs, filamin induced a pronounced drop in phase transition cooperativity, clearly showing the disturbing influence of filamin on the lipid packing and order which suggest at least partial incorporation of the protein into the lipid bilayer. Mixed DMPC/DMPG LUVETs showed a linear decrease of the main-phase transition enthalpy, ΔH and a significant shift in temperature, T_m for the solidus, T_s and liquidus, T_l lines with increasing mole fractions of filamin.

Filamin-lipid interaction of uncharged and negatively charged lipid monolayers in presence of filamin were examined by the film balance method. Recording time/area diagrams at constant pressure showed a marked effect with (DMPG) and a less pronounced effect with (DMPC), both indicating insertion of filamin into lipid monolayers.

Lipid membrane insertion of filamin was confirmed by lipid photolabelling. Using the lipid analogue [¹²⁵I] TID-PC, which selectively labels membrane-embedded hydrophobic domains of proteins, we showed that filamin partially inserts into the hydrophobic bilayer of liposomes.

Tu-101 MOLECULAR ANALYSIS OF MUTATIONS IN CONGENITAL SUCRASE-ISOMALTASE DEFICIENCIES

Joke Ouwendijk^a, Kitty Moolenaar^b, Jack Fransen^a, Leo Ginsel^a, Hassan Naim^b.

^aDept. of Cell Biology and Histology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. ^bInstitute of Microbiology, Heinrich-Heine-University, 40225 Düsseldorf, Germany.

Previous investigations of congenital sucrase-isomaltase deficiency (CSID) have lead to the identification of five phenotypes with respect to transport competence, polarized sorting and/or enzyme function of sucrase-isomaltase (SI) (1,2). Two of these mutant phenotypes, phenotype II (i.e. accumulation in the Golgi) and phenotype IV (i.e. missorting to the basolateral membrane) are of particular interest in the analysis of motifs implicated in proper transport of SI to the apical membrane. In the phenotype II case we describe here, SI accumulates as a mannose-rich precursor on one side of the Golgi apparatus and, in contrast to control brush border proteins (aminopeptidase N and lactase-phlorizin hydrolase), is not transported to the brush border membrane. These results indicate that the impaired transport of SI in this phenotype is not due to a general cellular defect and that the transport incompetence is restricted to SI. For this reason we isolated the SI cDNA of this patient by reverse-transcriptase-PCR technology and identified a mutation in a region of the sucrase subunit. This region is highly conserved between sucrase and isomaltase from different species, lysosomal α -glucosidase and *Schwannomyces occidentalis* glucoamylase (3). The mutation is currently being analyzed.

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Spectrin is associated with the plant plasma membrane

Tu-103

A.M.C. Emons¹, H. Kieft¹, N.C.A. de Ruijter¹, A. Sonesson², S. Widell²

¹Department of Plant Cytology and Morphology, Wageningen Agricultural University, Hollandseweg 4, 6706 BD Wageningen, The Netherlands

²Department of Plant Physiology, University of Lund, P.O. Box 7007, S-220 07, Sweden

Spectrin-like proteins were identified in plant cells using commercially available antibodies directed towards animal spectrin. With three different antibodies positive reactions were found with cells, tissues and membrane fractions of various origins.

Native plant proteins separated using isoelectric focusing or SDS-treated plant protein contained spectrin-like antigens. Such proteins were enriched in the plasma membranes as judged by Western blotting of cauliflower inflorescence membranes separated using SDS-PAGE. This distribution is similar to what earlier has been found with actin (Sonesson and Widell, Protoplasma, 177, 45-52, 1993).

Inside out plasma membrane vesicles of cauliflower inflorescences were immuno-gold labelled for transmission electron microscopy. Only a low percentage of inside-out vesicles was found labelled. However, *in situ* labelling of cauliflower inflorescences showed that only cells in the meristematic areas were labelled, which is only one tenth of the cauliflower peel used for the preparation of the vesicles.

We conclude that spectrin-like proteins are present at the cytoplasmic side of plant plasma membranes.

Tu-102 THE INFLUENCE OF THE INTRACELLULAR POLYOL CONTENT ON THE VIABILITY OF XYLOSE UTILIZING YEASTS DURING THE DEHYDRATION REHYDRATION PROCESS.

I.Krallish^a, H.Jeppsson^b, A.Rappoport,^a B.Hahn-Hägerdal^a.

Institute of Microbiology and Biotechnology, Latvian University, LV-1067, Riga, Latvia^a; Department of Applied Microbiology, Chemical Center, Lund Institute of Technology, Lund University, P.O. Box 124, S-22100 Lund, Sweden^b

During evolution microorganisms have developed different mechanisms allowing them to maintain their physiological activity and adapt to sudden changes of environment. Yeasts respond to short-term salt stress by accumulation of compatible solutes such as glycerol and other polyols that are supposed to counteract the reduced water activity of the surrounding medium.

We have studied the relationship between extra- and intracellular accumulation of polyols in different xylose-utilizing yeasts and their viability after slow dehydration. The viability of cells was determined using fluorescent microscopy. Xylose-utilizing yeasts with high(*Pachysolen tannophilus* CBS 4044, *S.cerevisiae* S600) and low(*Pichia stipitis* CBS 5667, *Pichia stipitis* CBS 6054, *S.cerevisiae* H 158) possibility to excrete xylitol in the surrounding medium were compared in polyols accumulation and viability after dehydration.

The accumulation and excretion of xylitol and other polyols depend on the genetic characteristics of yeast strain and on the carbon source in the growth medium. After process of dehydration intracellular polyols content was usually increased for most of xylitol accumulating yeasts. The results indicate that the accumulation of polyols in xylose-utilizing yeasts can be one of the protecting factors against the water stress imposed by drying and in some extent improve their viability during the dehydration-rehydration process.

MODIFICATION OF ANKRYIN IN THE EGG CYTOSKELETON MAY PRECEDE AN EVOLUTIONARY CHANGE IN ASCIDIAN DEVELOPMENT

W. R. Jeffery^{ab} and B. J. Swalla^b

^aSection of Molecular and Cellular Biology, University of California, Davis, CA 95616 and ^bBodega Marine Laboratory, Bodega Bay, CA 94923 USA

Most ascidians develop indirectly via a tadpole larva. However, a few ascidian species have eliminated the larva and develop directly into an adult. Direct development has evolved multiple times in the Family Molgulidae. Ascidian eggs contain a membrane cytoskeletal domain consisting of actin filaments, intermediate filaments, and a 58 kD protein (p58), which is segregated into the prospective larval muscle cells during embryogenesis. The egg cytoskeletal domain and p58 are lost or reduced in titer in eggs of direct developing species which have evolved independently. In the direct developing species, p58 is present in young oocytes but fails to be localized in the cortex and disappears later during oogenesis. To investigate the mechanisms underlying changes in p58 localization, we have determined the distribution of the membrane-cytoskeletal protein ankyrin in eggs of indirect and direct developing ascidians. A vertebrate ankyrin antibody reacted with a single 210 kD polypeptide in ascidian eggs. Immunofluorescence microscopy showed that ankyrin is localized in the oocyte cortex during oogenesis, segregated into the future posterior region of the embryo after fertilization, and partitioned primarily to the larval muscle cells during cleavage. This pattern of maternal ankyrin expression was observed in indirect developing species in three different ascidian families (Asciidae, Pyuridae, and Styelidae). In contrast, a dramatic change in the expression of maternal ankyrin was observed in six species of molgulid ascidians. Ankyrin was present in the cytoplasm of young oocytes but did not localize to the plasma membrane and disappeared in mature eggs. The modified pattern of ankyrin expression was observed in direct and indirect developing molgulids. These results suggest that maternal ankyrin may have been lost as a preadaptation to p58 modification and the evolution of direct development in ascidians.

EXPRESSION OF α -SMOOTH MUSCLE ACTIN-LIKE PROTEIN IN SOME METAZOA.

Tu-105

F. Simoncelli^a, F. Panara^a, I. DiRosa^a, A. Fagotti^a, J. Vandekerckhove^b, C. Chapponnier^c, G. Gabbianni^c and R. Pascolini^a

^aInstitute of Comparative Anatomy, University of Perugia, Italy; ^bUniversity of Ghent, Ghent, Belgium; ^cDept. de Pathologie, Université de Genève, Switzerland.

Using the anti- α SM-1 monoclonal antibody (Skalli et al., J. Cell Biol., 103, 2787-2796, 1986) as α -smooth muscle (aSM) actin probe, we analyzed its immunoreactivity in tissues of animals of different evolutive levels with both western-blotting and immunocytochemical methods. The data obtained provide new insights about smooth muscle actin phylogeny.

Biochemical and immunocytochemical results demonstrated that a positive immunoreactivity was detected in smooth muscle cells of the ectothermic vertebrates tested with the exception of teleosts. In addition, the embryos of the chordate invertebrate ascidians were also stained. Among other invertebrates, crayfish and freshwater planarians showed a positive reaction. Using the synthetic peptide Ac.(E)EED, which blocks the anti- α SM-1 binding to mammalian α SM actin, we observed that a similar blocking effect was exerted against all specimens showing anti- α SM-1 positive immunoreactivity.

These results suggest that an α SM actin-like protein, with an N-terminal sequence of Ac.(E)EED emerged early during animal evolution and indicate a functional role for this phylogenetically conserved epitope.

Tu-106

SPECTRIN EXTRACTIBILITY FROM ERYTHROCYTE IN RAT MORRIS HEPATOMA 5123.

A. Waliszewska, A. Wierzbicki, J. Batko, H. Karon

Departament and Chair of Physiological Chemistry Medical Academy, H. Swiecickiego 6, 60-781 Poznan, Poland

Several abnormalities have been described in red blood cells from rats with Morris hepatoma 5123. This alteration could result from biochemical or structural abnormalities of the erythrocyte membranes. Spectrin is one of the most abundant proteins of the erythrocyte membranes and is believed to be a determinant of the erythrocyte shape and deformability.

In the present study we compared the extractability of spectrin from Morris hepatoma ghost with that of controls. After the extraction of spectrin from ghost with low ionic strength buffer, membrane proteins were examined using SDS-polyacrylamide gel electrophoresis.

We found a significant decrease of spectrin extractability from Morris hepatoma erythrocyte ghost when compared with that of controls. In addition, the rate of spectrin extraction using these conditions was slower in Morris hepatoma.

This result appears to be due to the fact that spectrin is more tightly associated with the membrane in Morris hepatoma.

Tu-107

THE CONTENT OF N-ACETYLNEURAMINIC ACID IN GLYCOPROTEINS OF ERYTHROCYTE MEMBRANES IN MORRIS HEPATOMA 5123 BEARING RATS.

J. Batko, A. Waliszewska, H. Karon,

Departament and Chair of Physiological Chemistry Medical Academy, H. Swiecickiego 6, 60-781 Poznan, Poland.

Changes in the content of N-acetylneuraminc acid in rat erythrocyte membranes at different stages of experimental tumor (Morris hepatoma 5123) development were examined. Its content was lowered on the 30th and 40th day after transplantation of the tumor cells, as compared to the results for normal healthy rats. As a result of the tumor growth, the content of N-acetylgalactosamine, galactose and mannose in rat erythrocyte membranes became lowered, whereas that of glucose remained unchanged. The content of fucose was raised at early stage of tumor growth, and remained at this high level till 40th day of the experiment.

Calorimetric studies of the structural transitions in new-born erythrocyte membrane

Tu-108 Matveev A., Akoev V., Tarakhovsky Yu. Bryukhanov V.

^aInstitute of Theoretical and Experimental Biophysics, Pushchino, Altai Medical Institute, Barnaul, Russia.

The mechanisms underlying the functioning of new-born erythrocyte membranes are poorly understood. Using differential scanning microcalorimetry and freeze-fracture microscopy, a comparative study of the structure of erythrocyte ghost membranes from 9 new-born and 9 adult donors in phosphate buffer, pH 7.4, and 60 and 310 mosm has been carried out. Melting of new-born membrane suspension in 60 mosm exhibits 4 thermotropic transitions (A, B, C, D) with maxima at 51°C, 59°C, 65°C and 81°C, which is by 1.5-2 degrees higher than the temperatures of corresponding transitions in membranes of adult donors. At 310 mosm, of 5 transitions (A, B₁, B₂, C, D), only the C transition is shifted to 69-70°C. A strong variation in the intensity ratio for the A, B₁, B₂ and C transitions was found. The freeze-fracture study revealed no alteration in the fracture surfaces of new-born erythrocyte membranes. The microcalorimetric data suggest that the thermotropic properties of new-born erythrocyte membranes differ from those of adult donors. This is due to differences in structural organization of protein domains of the spectrin membrane skeleton. The differences in the functional properties of erythrocytes from neonatal and adult donors are probably determined by structural features of protein domains of the new-born erythrocyte membrane skeleton.

IMMUNOLOCALIZATION OF SERTOLI CELL

Tu-109 CYTOSKELETON IN A POECILIID FISH

M.I. Arenas, M.P. De Miguel, B. Fraile, R. Paniagua.

Department of Cell Biology and Genetics. University of Alcalá de Henares, Madrid, Spain.

Different proteins were visualized at electron microscopy level by colloidal gold labelling in the Sertoli cell of *Gambusia holbrooki*: actin, tubulin, vimentin, desmin and cytokeratins. Actin is present adjacent to the plasma membrane, in both the apical and basal cytoplasm, becoming more abundant in the apical zone with the progression of the spermatogenesis. Tubulin is scarce in the cytoplasm of this cell and its location is not variable along the spermatogenesis. Vimentin is restricted to the transformed Sertoli cells which form the efferent ducts. Similar localization of desmin was observed. Cytokeratins were not localized in the Sertoli cell of this fish.

IMMUNOCYTOCHEMICAL STUDY OF

Tu-110 INTERMEDIATE FILAMENTS IN THE TESTIS OF *GAMBUSIA HOLBROOKI* (TELEOSTEI).

M.P. De Miguel, B. Fraile, M.I. Arenas, R. Paniagua

Department of Cell Biology and Genetics. University of Alcalá de Henares, Madrid, Spain.

Desmin, vimentin and cytokeratins were localized by colloidal gold labelling at the electron microscopy level in the testis of the mosquito fish. Germ cell line is lack of these proteins; in contrast, these proteins are abundant in the interstitial cells. Desmin is present in the blood vessels, tubule boundary cells, fibroblasts, melanocytes and Leydig cells. Vimentin is localized in the same cellular types except the endothelium of the blood vessels, being more abundant in the tubule boundary cells. In the efferent ducts cells vimentin is also present. Cytokeratins are exclusively visualized in the endothelium of the blood vessels.

IMMUNOCHEMICAL STUDY OF THE EXPRESSION AND LOCALIZATION OF DYSTROPHIN-ASSOCIATED GLYCOPROTEINS DURING REGENERATION OF SKELETAL MUSCLE

Tu-111 R. Vater, M.J. Cullen and J.B. Harris

Muscular Dystrophy Research Laboratories, Newcastle General Hospital, Westgate Rd, Newcastle upon Tyne, NE4 6BE, UK

The linkage between dystrophin and the plasma membrane (PM) of skeletal muscle fibres has been reported to be mediated by a complex of 2 dystrophin-associated proteins (intracellular 59-DAP, integral 25-DAP) and 4 dystrophin-associated glycoproteins (integral 35-, 43- and 50-DAGs, extracellular 156-DAG) [1,2], and is known as the dystrophin-glycoprotein complex (DGC). The function of the DGC, however, has not yet been resolved. Considering its physical position linking the actin cytoskeleton to the basal lamina via dystrophin and the 156-DAG, the DGC could provide structural support to the PM, but it may have far more diverse functions such as signal transduction and regulation of calcium concentration. In Duchenne muscular dystrophy (DMD) and the *mdx* mouse, where dystrophin is absent, 80-90% of the DAG components are also lost. In severe childhood autosomal recessive muscular dystrophy (SCARMD) only 50-DAG is absent, yet this is associated with severe necrosis of the myofibres. Together these observations imply that the DGC has a critical function in the normal physiology of the muscle cell. We have shown by immunofluorescence and immunogold labelling that in regenerating rat skeletal muscle, the DAGs are expressed approximately 2 days in advance of dystrophin. This implies that the integration of the DAGs into the PM is independent of the expression of dystrophin, but that the presence of the DAGs may be necessary for the normal localisation of dystrophin and the function of the DGC as a whole.

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CYTOSKELETAL ELEMENTS ARE INVOLVED IN ERYTHROCYTE GHOST SURFACE MOTILITY

Tu-112 A.Krol, M.Grinfeldt, V.Malev

Laboratory of Cell Physiology, Institute of Cytology, Russian Acad. Sci., St.Petersburg, Russia

Previously we have suggested that the spontaneous fluctuations of the erythrocyte surface are caused by the membrane skeleton dynamics (Biol. Mem. 6, 701, 1993). Therefore we have continued to study the role of the cytoskeleton in the human red cell membrane undulations by applying cytoskeleton-binding proteins to permeable hypotonic ghosts. We have found that S1-fragment of skeletal myosin significantly suppresses the membrane undulations, but G-actin solutions restore them up to the initial level. The effect of S1 resulted from its binding to some skeletal component of the erythrocyte membrane, but possibly not to F-actin, since the threshold concentration of S1 is much greater (200 nM) than binding constant K_d of S1 to F-actin (1 nM). In our opinion, S1 exerts its effect most likely by binding to protein 4.1 ($K_d \approx 100$ nM). We also tried DNaseI, which complexes specifically with pointed ends of actin protofilaments in hypotonic ghosts. Preliminary data show that DNase inhibits surface undulations in the ghosts, but the effect is reversed by cytochalasin D, the drug binding to the barbed end of the protofilament. Though an explanation of the above effects is not quite clear and requires the further investigations it is obvious that actin ATPase suppressed by DNaseI and activated by cytochalasin D is involved in the generation of the erythrocyte ghosts surface undulations.

Tu-113 DYNAMIC CHANGES OF THE CYTOSKELETON
IN XENOPUS L. DURING OOGENESIS
P. Pokorný, J. Paleček,
H. Jiřincová, N. Česková

Dept. of Devel. Biol., Fac. of Sci. Charles Univ.
128 44 Prague 2, Czech Republic

The process of converting an egg to a multicellular organism often begins with polarization of information within the oocyte. Understanding how cell establish differentiated internal structures and overall morphologies is one of the major goals of cell biology. The cytoskeleton plays an important role in maintaining such assymetries. Anti-tubulin, - vimentin, - spectrin and - tropomyosin antibodies and indirect immunofluorescence microscopy were used to visualize dynamic changes of these cytoskeletal patterns within the serial sections from ovarian oocytes of *Xenopus*. The histological sections were prepared after several different immunocytochemical procedures, including of different combinations of aldehydic or alcoholic fixatives, detergents, temperature, drugs and embedding media. Dynamic changes of the cytoskeletal structures varied from a "simply" organized cytoplasmic, cortical and perinuclear arrays (previtellogenic oocytes) which become progressively more complex and polarized (vitellogenic oocytes). Bilateral symmetry of the fully-grown oocyte is thus reflected by morphology of the cytoskeletal system. The obtained results will be discussed.

Tu-115 CHANGES IN IMMUNOCHEMICAL LOCALIZATION OF CYTOSKELETAL PROTEINS DURING CAPACITATION AND INDUCED ACROSMOME REACTIONS
aM. Zachlederová, bJ. Pěkníková,
aJ. Paleček

^aDept. of Devel. Biol., Fac. of Sci. Charles Univ.
128 44 Prague 2, Czech Republic, ^bInst. of Mol.
Gen. Acad. of Sci. of The Czech Rep. Videaňská
1083, 142 20 Prague 4, Czech Republic

The spermatozoon is one of the most highly specialized of all mammalian cells and exhibits therefore extraordinary structural compartmentalization that is related to the presence of cytoskeletal proteins. The fixed boar and human spermatozoa were used for the visualization of cytoskeletal dynamic changes during capacitation and after induction of acrosome reaction by means of immunofluorescence and confocal microscopy. Monoclonal antibody against acrosin was used for detection of acrosomal exocytosis and panel of different monoclonal antibodies against alpha- and beta-tubulin, MAP-2, tropomyosin, spectrin, vimentin, cytokeratin and vinkulin were used for finding the role of cytoskeleton during this process. The most progressive changes were observed in the reorganization of tubulin (total absence in the end of acrosomal reaction) and spectrin (accumulation in the tip area of the sperm head) within the acrosomal region of the spermatozoon. The obtained results suggest the direct role of microtubules in induced acrosome reaction.

Tu-114 CHARACTERIZATION OF ENDOCYTIC PROCESSES IN XENOPUS OOCYTES USING ELECTRON DENSE TRACERS, PHORBOLESTER AND CYTOSKELETAL TOXINS

H.-P. Richter, A. Hansen and *K. Aktories
Departments of Physiology and Pharmacology*,
Saar-University, D-66421 Homburg/Saar

Oocytes and eggs are used to study endocytic processes during cell differentiation. Oocytes show a basal uptake of 2-5 nl of external fluid per oocyte·h, when oolemma is internalized via receptor-mediated pinocytosis or via unspecific fluid-phase endocytosis. The rate of [³H]inulin internalization increases to 5-12 nl/oocyte·h after treatment of the oocytes with the phorbol ester PMA. Stimulation by progesterone induces a transient burst of vesicle internalization of ca. 6 nl/oocyte within 1-2 h before GVBD.

Using EM tracers we morphologically followed the specific pathway of the internalized vesicles after application of ferric particles conjugated to the nutritive vitellogenin, or after incubation of the oocytes with horseradish peroxidase or with cationized ferritin. Both the PMA and progesterone cause a flattening of the oocyte surface. In addition, PMA dramatically perturbs the cortical cytoplasm with time: light microscopy shows depigmentation as well as pigment concentrations at the animal hemisphere, and electron microscopy detects clustering of cortical vesicles, strands of cytoskeletal elements and organelle-depletion. This PMA effect is reduced (i) after pre-injection of the oocytes with the *Clostridium botulinum* exoenzyme C3 or (ii) after incubation of the oocytes with specific inhibitors of the PKC or (iii) after application of Cytochalasin D.

The activated G-protein Rho A^{Val14}, applied together with progesterone, accelerates the resumption of meiotic maturation from oocytes to fertilizable eggs. These findings indicate that Rho proteins are probably involved in the regulation of actin-mediated endocytosis in *Xenopus* oocytes.

Support by Deutsche Forschungsgemeinschaft (SFB 246, B 10*, C5)

Tu-116 REACTION OF CELL MEMBRANES AND GROUND CYTOPLASM TO TREATMENT WITH XANTHOTOXIN
A. Zobel^a, Ch. Ashby^b, A. Gawron^b, K. Głowniak^c

^a Chemistry Department, Trent University, Peterborough, Ontario Canada; ^b Department of Cell Biology, Maria Curie-Skłodowska University, Lublin, Poland; ^c Pharmacognosy Department, Medical Academy, Lublin, Poland

Furanocomarin xanthotoxin is used in combination with UV-A in treatment of various skin diseases such as psoriasis, vitiligo and a disease of accelerated epidermal cell proliferation. The photochemotherapeutic effects of furanocoumarins for the skin are thought to arise from their ability to form cyclobutane-type adducts with pyrimidine bases of nucleic acids after UV radiation. Investigations of last years have shown that also membrane constituents may be a biological target in the photosensitizing action of furanocoumarins.

In present studies we found that photoactivated xanthotoxin altered plasma membranes permeability and caused increase of concentration of lysosomal enzyme - acid phosphate outside the cells. However, activity of this enzyme was related with presence of vesicles at diameter about 0.45-0.80 μm. These results indicate that photoactivated xanthotoxin disturb structure of cell membranes and caused segregation of cytoplasm.

Formation vesicles can be induced either by an oxygen-dependent mechanism (lipid peroxidation, formation of cross-links in proteins) or an oxygen - independent mechanism, (which is due to a direct photoreaction between xanthotoxin and unsaturated fatty acids) and indicates that the effect on the cell membrane plays an important role in the mechanism of the action of xanthotoxin on the cells.

Tu-117 MICROTUBULES AND CYTOPLASMIC REORGANIZATION DURING FIRST CELL CYCLE OF XENOPUS EGG.
G.A. Ubbeis^a, J. Pajecek^b

^a Hubrecht Laboratory, 35 84 CT, UTRECHT, The Netherlands, ^b Dept. of Devel.Biol., Fac.of Sci. Charles Univ., 128 44 Prague 2, Czech Republic

In the fertilized egg of *Xenopus* I. the bilateral symmetry is epigenetically determined during the first cell cycle, by cytoplasmic shifts and translocation of the cortex over the cytoplasm, due to activities of the egg's cytoskeleton. By injecting the fluorescent dye trypan blue (TB) into the dorsal lymph sac of *Xenopus* females, a particular group of yolk granules was pre-labelled during oogenesis. With CLSM we studied in-vivo and in-vitro, the distribution of fluorescently labelled yolk in wild-type (= pigmented) and albino (= unpigmented) eggs from fertilization till first cleavage, and determined the time and duration of the cortical rotation. To test whether cortical or cytoplasmic microtubules are involved in the cytoplasmic rearrangements, anti-tubulin immunofluorescence staining was applied on histological sections, groups of eggs were also treated in toto with different doses of UV. We will discuss the role of the cytoskeleton and yolk relocations in the establishment of the prospective embryonic axes.

Tu-119 ELECTRON-MICROSCOPY OF THE INFLUENCE OF DIFFERENT DOSES OF GAMMA RADIATION ON STRUCTURE AND MORPHOLOGY OF RAT SPERMATOZOA

A. Andreychenko, O. Olchenko, V. Ruban
Biology faculty of the Ukrainian medical university, Kiev

With the purpose of a study of the influence gamma radiation on the rats' spermatozoa and an exposure of the possible changes their morphological structure we extracted an epididymis from the ripe males and irradiated it by gamma rays (cobolt -60) in the different doses from 0,1 to 0,5 Gy. The preparations were investigated with the help of the electron microscopes JEM -35 and PJ -100.

We originally studied the cytological preparations with the help of a light microscope and we exposed a definite correlation between a dose of the gamma irradiation of the spermatozoa and an appearance of the morphological changes and the reconstructions in its structure. With the help of a scanning electron microscopy we managed to show that a reinforcement of the mobility of the plasmatic membranes and a superficial lipide matrix are the basis of the radial defeat of the spermatozoa. As it turned out the gamma irradiation quicken of a process of a flow of the lipide matrix from the surface of the spermatozoon, which with a rise of the dose of the irradiation assume a local character and assume likeness with a necrosis. As a result we observe a thinness of the separate morpho-anatomical structures of the spermatozoa and its following death.

Tu-118

ULTRASTRUCTURAL RESPONSES OF PLANT CELLS TO ENVIRONMENTAL STRESSES
M. Čiamporová^a, M. Moustakas^b, Ouzounidou^b and I. Tsekos^b

^aInstitute of Botany, Slovak Academy of Sciences, 842 23 Bratislava, Slovak Republic

^bInstitute of Botany, Aristotle University of Thessaloniki, 540 06 Thessaloniki, Greece

Abiotic stresses can induce a variety of ultrastructural responses which correspond with disturbance of cellular physiological functions. These responses may depend on the type and strength of a stress, on plant species and also on the type and age of the treated cells. Electron micrographs of root meristem and leaf mesophyll cells exposed to several kinds of non-lethal stress conditions (water deficit, salinity, cold, heat, heavy metals), have shown that the continuity of cell membranes was preserved and the cellular compartments were not injured. Several processes of adaptation are indicated by the modifications of the submicroscopical morphology of cell components, e.g. synthesis of stress proteins (by structural organization of nuclei, ER, Golgi apparatus, and ribosomes), solute accumulation and osmotic adjustment (by plastid and vacuole responses), and toxic metal sequestration (by structural changes in vacuoles and cell walls).

Tu-120 HIGHER-ORDER ORGANIZATION OF CHROMATIN FROM TRANSCRIPTIONALLY ACTIVE AND INACTIVE CELLS

V.I.Vorob'ev, E.V.Karpova, T.N.Osipova

Institute of Cytology of the Russian Academy of Sciences, St.-Petersburg, 194064, Russia

Comparative studies of chromatins differing in transcriptional activity and in the internucleosomal (linker) DNA length represent a fruitful approach to the problem of higher-order chromatin structure. Chromatins were prepared from the nuclei of pigeon brain cortical neurones, rat thymus and sea urchin sperm characterized by linker DNA sizes of 20, 50 and 100 bp respectively. Oligonucleosomes of different chain length were isolated from nuclease digested nuclei and their hydrodynamic and optical properties have been studied by the methods of sedimentation velocity and circular dichroism. The behaviour of nucleosome oligomers in the dependence on the number of nucleosomes in the chain and on the ionic strength has been analysed. The results obtained allow one to distinguish different levels of nucleosome organization in chromatin and to evaluate the role of linker proteins and the linker DNA size in a sequential salt-induced nucleosome packing in chromatin. It was shown that hydrodynamic behaviour of oligonucleosomes at low ionic strength can be well described by the model of three-dimensional zig-zag chain with similar diameter, DNA packing ratio growing with increase of linker DNA length. With increase of ionic strength zig-zag shaped nucleosomal chain is condensed into a two-start double superhelix with closely arranged nucleosomes and linker DNA loops packed inside the superhelix.

Tu-121 INTERACTION BETWEEN SPERM CELLS AND EXOGENOUS DNA: SPERM MEDIATED GENE TRANSFER

M. Lavitrano^c, V. Lulli^a, B. Maione^c, M. Zanic^c, M. Francolini^a and C. Spadafora^b

^aIstituto Tecnologie Biomediche, CNR, Via Morgagni 30/E, 00161, Rome, Italy, ^bIst.Superiore Sanità, Rome, Italy, ^cDept. Medicina Sperimentale, Università "La Sapienza", Rome, Italy.

Mammalian sperm cells, depleted of seminal fluid, show an inherent capability to take up foreign DNA. In mice 15-20 % of the sperm bound DNA is further internalized into sperm nuclei following a specific pathway. Protein factors modulating the process of nuclear internalization have been identified and partially characterized. A portion of the nuclear -internalized exogenous DNA is constantly found in tight association with the sperm nuclear scaffold. Matrix-associated exogenous DNA shows an altered restriction pattern reflecting the occurrence of specific rearrangements. Exogenous DNA bound to sperm cells can be transferred to oocytes during the process of fertilization causing a genetic transformation of the embryos. By this method, using the pSV2CAT plasmid, we have obtained transformed mouse, swine and bovine embryos as well as transgenic mice and pigs. It is noteworthy that, while the production of transgenic mice using sperms from epididymus showed broad fluctuations among different experiments (0 - 80 %), transgenic embryos from both pig and bovine species, were obtained with reproducible efficiency, using ejaculated semen (16 %). Moreover a different degree of efficiency was repeatedly observed when various DNA constructs were tested in parallel experiments. This suggests that specific DNA sequences may play a role driving the transformation process.

Tu-122 HEPARIN-BINDING PROTEINS ON MAMMALIAN SPERMATOZOA: BIOCHEMICAL CHARACTERIZATION, CELLULAR TOPOGRAPHY, AND THEIR FATE DURING SPERM CAPACITATION

J.J. Calvete^a, L. Sanz^a, Z. Dostálová^a, K-H. Scheit^b, F. Sinowatz^c, W. Amselgruber^c, and E. Töpfer-Petersen^a

^aInstitut für Reproduktionsmedizin, Tierärztliche Hochschule, Bünteweg 15, 30559-Hannover, Germany; ^bMax Planck Institut für Biophysikalische Chemie, Göttingen, Germany; ^cLehrstuhl für Tieranatomie II, Universität München, Germany.

Heparin-binding proteins (HBPs) present in the male sex accessory gland secretions (seminal plasma) become attached to the sperm surface at ejaculation. Their ligands are proteoglycans with heparin-like glycosaminoglycan side chains secreted by the epithelium of the female reproductive tract, and which, in most mammalian species investigated, stimulate sperm capacitation and/or the acrosome reaction. We have isolated and characterized the major HBPs on sperm from different mammals: boar (12-15 kDa), bull (12-30 kDa), stallion (25-30 kDa), and man (28 and 80 kDa). The seminal vesicles are the major, but not the sole, site of synthesis of HBPs. Using monospecific polyclonal antibodies we have quantitated the number of individual HBPs/spermatozoa at different stages of the sperm's life. These studies show that a large population of the HBPs remains sperm-associated upon *in vitro* capacitation. However, our results show that structurally and immunochemically the HBPs belong to different protein families and that their topography is not conserved on sperm from the four species investigated. These data suggest the possibility that HBPs could represent species-specific capacitation factors.

(Financed by Grants DFG Tö 114/1-2 and BMFT 01KY9103)

Tu-123 THE PRODUCTION OF TRANSGENIC SHEEP BY SPERM MEDIATED GENE TRANSFER METHOD

Sun Yong Xin^a, Chen Dong^a, Guo Zhi Qin^a, and Chen Yong Fu^a

^aNational Agrobiotechnology Lab, Beijing Agricultural University, Beijing 100094, P.R. China; ^bAcademy of Animal Science, Urumqi 830000, Xinjiang, P.R. China

The capacitated sperm cells had been demonstrated having the DNA carrying ability after removal of the coated glucoprotein of acrosome by our experiment on Xin-Jiang Merino sheep. It had great relation with the completion of capacitation, the concentration of donor DNA, the medium used and the incubated condition.

The sperm cells capacitated by heparin incubated with 10 µg/ml cloned circular DNA (ovine MT promoter and bovine Growth Hormone fusion gene, OMT/bGH) for 30 min. Above cells were used to fertilize the sheep eggs *in vivo* by artificial insemination. The *in vitro* studying result of scanning and transmission electron microautoradiograph showed that the labelled DNA had transferred into the head of about 13% sperm cells but was absent in the cells without capacitation disposal or the capacitated cells killed by UV exposure. Seven transgenic progeny and three positive hereditary lambs were gotten by the identification of Southern blot and PCR amplification. The efficiency of transgene was about 2.5%. The expression level of donor OMT/bGH gene in serum ranged from 35 to 150 ng/ml and the average body weight of transgenic sheeps was about 5Kg weighter than the control.

Tu-124 DNA UPTAKE BY MICE AND TOAD SPERM CELLS

J. Mácha^a, D. Štursová^a, V. Habrová^a, M. Takáč^b, and J. Jonák^b

^aDept. of Devel.Biol., Fac. of Sci. Charles Univ. 128 44 Prague 2, Czech Republik, ^bInst. of Mol. Gen. Acad. of Sci. of The Czech Rep., Flemingovo n. 2, 166 37 Prague 6, Czech Republik

This paper deals with study of the appropriate conditions for DNA uptake by sperm cells of *Xenopus laevis* and mice.

Plasmid pAPrC (12,4 kb) linearized by Sal I and labelled on 5' end with 32 P was incubated with sperm cells in 60 mM (toad) or 100 mM (mice) NaCl, 15 mM Tris-HCl pH 7,6, 1 mM KCl and 0,1 percent BSA. The sperm cells were then filtered, washed and DPM was measured. In a parallel experiment the incubation was followed by 2 min. treatment with 4 µg DNase per ml, so that only internalized DNA remains. The incorporation of total DNA reached plateau in 20 min. for both mice and toad. In this time 72 percent (mice) and 64 percent (toad) DNA was internalized. The DNA internalization decreased in 60 min. at *Xenopus*, revealing sperm damage. Calcium, magnesium and EDTA (1 mM) had only slight effect, EDTA rised DNA uptake in all cases. Both total and internalized DNA uptake depends on its concentration with the usual Langmuir course. Pyruvate, glutamate and antimycin had no effect on the DNA internalization.

Tu-125 TRANSFER OF ROUS SARCOMA VIRUS DNA TO OVA BY XENOPUS LAEVIS SPERMATOZOA

J. Jonák^a, V. Habrová^b, M. Takáč^a
J. Mácha, M. Reiniš^a and H. Pokorná^b

^aInstitute of Molecular Genetics,
Academy of Sciences of the Czech Republic and
^bDepartment of Physiology and Developmental
Biology, Faculty of Science, Charles University,
Prague, Czech Republic

Fertilization of X. laevis eggs with spermatozoa preincubated with plasmid pAPrC carrying the complete Rous sarcoma virus (RSV) DNA induced developmental malformations (head and eye defects, oedemas, axial deformations etc) in 25-30% of 7-day frog embryos. Immunohistochemical analysis of tissue sections from defective animals revealed aberrations in myotomal structures, disintegration of their actin filaments and increased expression of pp60^{src} protein in myoblasts and mesenchymal cells. The presence of v-src and RSV-long terminal repeat (LTR) sequences in embryonal DNA was detected by Southern blot and PCR analyses. X. laevis RNA strongly hybridized with a src-specific and a RSV-LTR-specific probe indicating extensive expression of the viral DNA. Plasmid DNAs without v-src gene (pATV9, pBR322) did not induce any changes in the morphogenesis of X. laevis. The results provide evidence that the cloned DNA form of RSV genome associates with frog sperm cells and may be subsequently carried into eggs during fertilization. Correlation between the defective morphogenesis and increased expression of the src gene in tissues suggests that RSV DNA may interfere with developmental programmes of the frog.

Tu-127 THE DEVELOPING XENOPUS LAEVIS BRAIN.

R. Vignali, B. Kašlar, M. Pannese*, M. Andreazzoli*, C. Pojo*, L. Menotti, E. Boncinelli¹ and G. Barsacchi.
Laboratori di Biologia Cellulare e dello Sviluppo, Università di Pisa, Italy; * DIBIT, Istituto Scientifico H.S. Raffaele, Milano, Italy

We analyzed the expression of four homeobox genes related to two *Drosophila* genes expressed in the developing fly head (*Ybx1*, *Ybx2*, *Xemx1*, related to *orthodenticle*; *Xemx2*, related to *empty spiracles*). These genes are all expressed in the developing rostral brain of a tailbud embryo, where their expression domains can be mutually compared. *Xemx2* is expressed in prosencephalon and mesencephalon, both dorsally and ventrally. The *Xemx2* expression domain is similar to that of *Ybx2*, which is contained within it; we have preliminary evidence of two forms of the *Xemx1* gene, which are differently expressed in the otic vesicle. The *Xemx2* expression domain is exclusively confined to the dorsal prosencephalon, and that of *Xemx1* is contained within it. During the period of regional specification in the anterior brain the expression domains of the four genes appear to be continuous regions contained within each other in the sequence *Xemx2*-*Ybx1*-*Xemx2*-*Yemx1*. The temporal expression pattern of these genes is also sequential: *Ybx2* is expressed first (stage 10), followed by *Yemx1* (st. 11), *Xemx2* (st. 18) and finally *Xemx1* (st. 26). These results are consistent with the data on the mouse and suggest a role for these genes in controlling regionalization within the anterior brain of vertebrates. The expression pattern of *Xemx2* appears to be particularly interesting since transcription from this gene is turned on in the Spemann's organizer region at the beginning of gastrulation. During successive stages *Xemx2* expression first becomes restricted to the anteriormost region of all three germ layers, and then persists at a high level only in the nervous system. This pattern raises questions on the possible role of this gene in determining the peculiar properties of the cells of the dorsal blastopore lip; on the possible relationships of *Xemx2* to other genes that are also expressed in this embryonal area; on its involvement in anterior neural induction. Microinjection experiments suggest that *Xemx2* plays a role in the early determination of anterior cell populations as well as in balancing the correct proportion between head and trunk structures.

UPTAKE OF EXOGENOUS DNA BY COCK SPERMATOZOA

Tu-126

P. Trefil^a, J. Mika^a, D. Slíva^b

^aResearch Institute of Animal Production, Prague 10 - Uhříněves

^bResearch Institute for Veterinary Drugs, Jílové near Prague

Transfer of foreign DNA into avian genome by spermatozoa is one of the hopeful method for the generation of transgenic chicken. First step to solve this problem is efficient association of foreign DNA with spermatozoa. The aim of this study was to find amount of plasmid for sufficient association with spermatozoa and investigate the influence of temperature on association of pKT3 with cock spermatozoa.

We found out that sufficient amount of the plasmid pKT3 for incubation with spermatozoa was 10-30 ng per 100 x 10⁶ spermatozoa. Association of plasmid with spermatozoa was time dependent (maximum 45 min of incubation). Higher percentages of association of plasmid with spermatozoa were detected after incubation of both forms of pKT3 at a temperature of 40°C. The presence of DNase I decreased the number of spermatozoa associated with pKT3 in both temperatures and forms except linear form incubated at 5°C.

NONSYNDROMIC CLEFT LIP AND PALATE:
EVIDENCE OF LINKAGE TO MICROSATELLITE

Tu-128 MARKERS IN 6P.

M.Tognon^a, F.Pezzetti^a, L.Scapoli^a, F.Carinci^b,
E.Padula^a and P.Carinci^c.

^aIst.Histol.Gen.Embryol.; ^bChair Maxillo-Facial Surg., Univ. FERRARA; ^cMaxillo-Facial Unit, S.Bortolo Hosp., VICENZA, Italy.

Nonsyndromic cleft lip with or without cleft palate (CLP) is a common craniofacial anomaly, whose etiology is unknown. Moreover, population's study showed that a large proportion of cases occur sporadically.

Recently, segregation analyses applied to CLP families indicated that an autosomal dominant/codominant gene (s) may cause clefting in cases.

Moreover, early studies suggested the associations of autosomal dominant CLP with HLA and F13A genes, which map on chromosome 6p.

In our study, microsatellite markers at loci D6S105, D6S109, D6S260, D6S89, EDN1 and F13A were amplified by PCR and then resolved on polyacrylamide gels.

Seventeen multiplex CLP families were analyzed with each marker. Linkage was tested using MLINK and LINKMAP programs. From these studies positive LOD score was observed.

Tu-129 ISOLATION AND CHARACTERISATION OF THE GENES THAT REGULATE TIP FORMATION AND AGGREGATE SIZE IN DICTYOSTELIUM

N.V. Zhukovskaya and J.G. Williams

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts., EN6 3LD U.K.

Dictyostelium displays regulative development and the tip that forms at the top of the aggregate has a very similar role to the organiser region of vertebrate embryos. By exerting a signalling property called tip dominance and so controlling the number of cells in any one aggregate, the tip dictates the eventual size of the fruiting body. We have isolated five, independent mutants in which every aggregate that is formed yields a multi-tipped structure. The mutants were generated by a method of insertional gene disruption called Restriction Enzyme Mediated Integration (REMI) that allows for the easy isolation of the affected gene. Based upon studies using a dominant inhibitor of the cAMP dependent protein kinase (PKA), we have developed a model for tip dominance wherein PKA plays a key role. Hence we believe that, by studying these mutants, we may discover a novel signalling pathway involving PKA.

EXPRESSION OF RENAL PODOCYTE CHARACTERISTICS UNDER PROLONGED PERFUSION CULTURE

Tu-130 S. Kloth, A. Schmidbauer, M. Kubitz, W. Röckl and W.W. Minuth

Institute of Anatomy, University of Regensburg, 93053 Regensburg, Germany

The development of the glomerulus starts with the inductive interaction of the embryonic collecting duct and the surrounding mesenchyme. The subsequent condensation of mesenchymal cells results in the formation of the renal vesicle, which represents the first visible nephron stage. The renal vesicle proliferates and changes its shape to form the S-shaped body (SSB). By means of the monoclonal antibody EnPo 1 first glomerular precursor cells can be detected within the lower part of the SSB (Kloth et al. 1992, *Differentiation* 52, 79). The following developmental steps give rise to the well known glomerular structures.

Frequently, it has been reported that podocytes show a high tendency to dedifferentiate under *in vitro* conditions. Thus, an organotypic culture system was used for our investigations on podocyte development. Renal cortex explants consisting of fibrous capsule, mesenchyme, collecting duct ampullae, developing nephrons and endothelial cells were prepared from neonatal rabbit kidneys. These explants were cultured without serum supplements for 14 days under permanent medium perfusion.

The EnPo 1 antigen could be detected during the whole culture period. Furthermore, as shown by kinetic experiments, the spatial organization of the podocyte precursor cells of the SSB changed, and a glomerulus-like structure developed within 3 days. Following conventional stationary culture only desintegrated, scattered EnPo 1 labelled cell clusters were found. We conclude that the organotypic environment is not sufficient to preserve antigenicity and developmental potency of podocytes, while the continuous exchange of culture medium resulted in excellent tissue preservation and typical antigen expression.

DIETARY FISH OIL ACTS AS PEROXISOME PROLIFERATOR IN THE MOUSE.

Tu-131 D. De Craemer¹, J. Vamecq² and C. Van den Branden¹
¹Human Anatomy, VUB, Laarbeeklaan 103, B-1090 Brussels; ²North France Center for the Study of Childhood Epilepsy, CHRU, F-59037 Lille.

Fatty acids play a regulatory role in the activity of peroxisome proliferator activated receptor (PPAR). This ligand-activated transcription factor binds to the peroxisome proliferator response element (PPRE), a specific DNA-sequence located upstream of genes encoding for peroxisomal beta-oxidation enzymes. Recently, it was shown that polyunsaturated fatty acids (PUFA), in a physiologic concentration, were as potent activators of PPAR as the most potent peroxisome proliferator Wy 14,643. We therefore investigated the effects of fish oil, which contains 57% PUFA, on the hepatic peroxisomes *in vivo*.

Fish oil was added to the diet of adult male mice for up to three weeks. A high concentration (10% w/w) of fish oil strongly induced (\pm 7.5 fold) peroxisomal beta-oxidation capacity in the liver; 0.8% fish oil increased it 1.5 fold, while 0.1% had no effect when compared to control animals. Only in mice fed 10% fish oil, catalase activity was increased (2 fold) and the number of peroxisomes was doubled.

These results indicate that *in vivo*, PUFA indeed act as peroxisome proliferators although their effect is but elicited at very high (above physiologic) concentrations. In addition, PUFA do not induce an as massive increase in peroxisomal number as do Wy 14,643 and other peroxisome proliferators.

Supported by NFWO and Onderzoeksraad VUB.

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TISSUE-SPECIFIC EXPRESSION OF HUMAN

Tu-133 GENES IN FETAL LIVER AND BRAIN

V. Dmitrenko, O. Garifulin, V. Kavsan

Institute of Molecular Biology and Genetics,
Ukrainian Acad. Sci., 150 Zabolotnogo St.,
252627 Kiev, Ukraine

Four cDNA libraries were used for our investigations. Two human fetal (23 weeks old) liver cDNA libraries in pBR322 plasmid and λgt10 bacteriophage vectors were constructed in our laboratory. One human fetal (26 weeks old) liver cDNA library was obtained from Clontech Laboratories. High density cDNA filters contained 20000 human fetal (17 weeks old) brain cDNA clones each were obtained from Reference Library, ICRF. Differential hybridization of cDNA libraries with human fetal liver, human fetal brain and human adult kidney cDNA probes revealed the subsets of transcripts: "liver-specific", "brain-specific", "house-keeping", "embryonic", "adult". The mitochondrial cDNA clones were identified by hybridization with mouse mitochondrial DNA probe. Some individual cDNA probes (e.g. serum albumin, gamma-globin) were used for identification of clones contained abundant transcripts that prevented repeated sequencing of those clones. The sequencing of cDNA inserts from different cDNA clones amplified by PCR directly from phage plaques picked into SM buffer was performed by Sanger method. Sequence analysis showed that the majority of investigated cDNAs was not represented in EMBL and Genebank databases.

TRANSCRIPTION FACTORS AP-1, Sp-1, AND

Tu-135 NF-κB ARE DOWN-REGULATED DURING MYOCYTE DIFFERENTIATION

S. Lehtinen, P. Rahkila, M. Helenius, A. Salminen.

Department of Cell Biology, University of Jyväskylä, Jyväskylä, Finland.

Changes in transcription factors AP-1, Sp-1, and NF-κB were analyzed by DNA-protein binding assays and immunoblotting during the differentiation of L6, C2C12, and rhabdomyosarcoma BA-Han-1C myocytes. Nuclear DNA-binding activities of AP-1, Sp-1, and NF-κB were down-regulated both in L6 and C2C12 myocytes (2% horse serum) and BA-Han-1C cells (suramin) 12 hours after switching the differentiation. Deoxycholate-activated total cytoplasmic NF-κB activity decreased during differentiation. Immunoblotting also showed the down-regulation of transcription factors. Myogenin expression, the master gene of muscle differentiation, was up-regulated 24 hours after the switch.

Cholera toxin, contrary to its down-regulation of myogenin expression, increased the binding activities of AP-1, Sp-1, and NF-κB. Okadaic acid also increased AP-1 binding activity in myocytes.

CELL TYPING BY MULTIVARIATE ANALYSIS OF GENE EXPRESSION

Tu-134

E. Spanakis

Institut d'Oncologie Cellulaire et Moléculaire Humaine,
129 rue de Stalingrad, F-93000, BOBIGNY, France.

What are mammary gland fibroblasts? How much, and in what, do they differ from, say, mammary epithelial cells or from fibroblasts of other organs? Are tumoral fibroblasts the same as the fibroblasts of normal tissues? Such questions interest both, developmental biologists and epidemiologists. If a phenotypic difference is due to a differential expression of one, or few genes then the search for reliable marker genes is worthy. If, on the other hand, there are fine quantitative differences in the expression of most genes between cell types, then random genes may provide little, though useful, bits of discriminatory information. Small differences in many genes may add up to a readily recognizable gene expression pattern. I describe a multivariate analysis of slot blot autoradiographic data on random transcripts that are generally related to growth, differentiation or cancer. This analysis makes it possible to confidently identify the developmental or pathological state of a cell population without having to examine cell-type-specific traits (which may not exist). Mammary fibroblasts and epithelial cells were classified as such with 100% success and fibroblasts from various pathological mammary tissues were so at rates far higher than any known single phenotypic marker can guarantee.

APPLICATION OF FUZZY SET ANALYSIS
IN SIMULATION OF HAEMOPOIETIC CELL**Tu-136 CHANGES**

J. Berger

University of Pardubice, Department
of Clinical Biology and Biochemistry, Stropo
Ltd., Strossova 57, 530 03 Pardubice, Czech
Republic

A good deal of biological information is inherently characterized by uncertainty. According to L...Zadeh a fuzzy set consists of objects and their respective grades of membership in the set. The grade of membership of an object, i.e. a cell in the fuzzy set is given by a subjectively defined membership function.

At a time point the lot of a haemopoietic cell is determined within a framework. The cell can (1) be released into blood, (2) mature without mitosis, (3) die, (4) come in the mitotic phase, or (5) be drawn into a pathologic process. The fuzzy set of haemopoietic cells is based on these five main cell categories. The actual grade of membership of a haemopoietic cell ranges from 0.0 to 1.0 where a value 1 denotes full membership.

The input of a haemopoietic cell into one cell category mentioned above is caused by one from five regulating effects. Simulating cell changes with the fuzzy logic we can use a system based on rules If (an effect) THEN (a result) to describe the cell change.

Tu-137

ANALYSIS OF THE REGULATORY ELEMENTS INVOLVED IN THE *Drosophila* VITELLINE MEMBRANE PROTEIN 32E GENE EXPRESSION.

Giuseppe Gargiulo, Franco Graziani*, Carla Malva*. Dipartimento di Biologia Evoluzionistica Sperimentale, Via Belmeloro 8, 40126 Bologna, Italy
 *Istituto Internazionale di Genetica e Biosifisica, CNR, Via Marconi 10, 80125 Napoli, Italy

Our previous studies on the *Drosophila melanogaster* vitelline membrane protein gene VMP32E have shown the existence of a spatial and temporal regulation of the VMP32E gene expression in different domains of the follicular epithelium (Gargiulo et al. 1991).

DNA sequences, extending from -465 to -39 bp. upstream the transcription initiation site are sufficient to direct specific expression of the VMP32E gene. Deletion analysis revealed the presence of at least two cis-acting regulatory elements (-465/-249, -135/-39). Both these regulatory regions are necessary to determine the specificity of the VMP gene expression in ovaries.

The functionally defined region, located between nucleotides -465 and -249 shares substantial sequences similarities with the CF1/Usp receptor DNA binding domain of *D. melanogaster* chorion s15 gene (Shea et al. 1990). We have analysed the potential binding of the CF1/Usp factor to the VMP gene promoter by means of an *in vitro* assay using a bacterial expressed CF1/Usp factor. The DNaseI protection footprint analysis shows a clear binding of the CF1/Usp in the VMP regulatory region. To investigate the transcriptional regulatory role of the DNA sequences harbouring the CF1/Usp DNA-binding domain in the VMP32E gene expression, we have replaced the CF1/Usp binding domain with pUC18 DNA polylinker sequences. The mutated DNA fragment was fused to heterologous basal promoter and reporter gene (hsp/LacZ) and used in transformation experiments. The transformant flies carrying the mutated CF1/Usp binding site show strong bias for staining in the ventral region, and little or no staining in the dorsal region. This novel and characteristic spatial pattern of staining suggests the existence of multiple cis-regulatory elements that allow expression in specific spatial domains of the follicular epithelium.

Gargiulo, G., Gigliotti, S., Malva, C., and Graziani, F. (1991) Mechanisms of Development, 35, 193-203.

Shea, M.J., King, D.L., Conboy, M.J., Mariani, B.D., and Kafatos, F.C. (1990) Genes & Development, 4, 1128-1140

Tu-138

REGULATION OF TISSUE SPECIFIC EXPRESSION OF ENOLASE ISOFORMS

A. Keller, N. Lamandé, M. Lucas

Laboratoire de Biochimie Cellulaire, CNRS URA 1115, Collège de France, F-75231 Paris, Cedex 05, France.

In higher vertebrates, the glycolytic enzyme enolase (EC 4.2.1.11) is found as homodimers and heterodimers formed from three subunits α , β and γ , encoded by distinct genes. As in many tissues, the $\alpha\alpha$ embryonic form remains the only expressed enolase isoform in liver, at the adult stage. Contrarily to what has been reported for the rising enzymatic activity, we observe a postnatal drop in α transcript levels of mouse liver. In contrast, we have demonstrated that the progressive transition which occurs during brain development, from the embryonic $\alpha\alpha$ enolase to the neuron specific isoforms ($\alpha\gamma$ and $\gamma\gamma$), is mainly controlled by the amounts of corresponding mRNAs. *In situ* hybridization studies reveal the coexpression of α and γ enolase genes in many neurons of rat brain, accounting for the high level of $\alpha\gamma$ hybrid (30%) extracted from adult brains. We further observe a differential expression of α and γ enolase genes in various neuronal populations of the brain.

During myogenesis, a transition occurs from α to β enolase gene expression. The β enolase gene is specifically expressed in the heart and all striated muscles as soon as they form. In hindlimb muscles, a large increase of β transcript and subunit levels occurs at fetal stages, followed by a postnatal increase at the time of fiber specialization, with a lower expression of this gene in slow fibers than in fast fibers. Pulse labeling experiments of newly synthesized transcripts in the presence of thiouridine demonstrate that the accumulation of β enolase mRNA in differentiating C2 myogenic cells is controlled by the amount of newly synthesized transcripts and suggest a transcriptional regulation.

The enolase gene family therefore appears as a useful model system to analyze the molecular mechanisms which govern cell and developmental stage specific gene expression.

Tu-140

THE RELATIVE CONTRIBUTIONS OF VARIOUS TRANSCRIPTION FACTORS TO THE TRANSCRIPTION OF THE MOUSE RIBOSOMAL PROTEIN L30 GENE

G. Sáfrány^a and R.P. Perry^b

^aNational Research Institute for Radiobiology and Radiohygiene, Budapest 1775, Hungary; ^bInstitute for Cancer Research, Fox Chase Cancer Center, Philadelphia, USA

The promoter of the mouse gene encoding ribosomal protein L30 (rpL30) contains binding sites for four different transcription factors: alfa (RFX-1), beta (GABP), gamma and delta (YY1, NF-E1, UCRBP). The relative contributions of these factors to the strength of the rpl30 promoter and the degree of synergism among the factors were investigated by transfection experiments with different mutant constructs. These mutations eliminated the binding of the appropriate transcription factors. Our results indicated that GABP and RFX-1 are the major determinants of the promoter strength in S194 mouse plasmacytoma and simian cos7 cells. The contributions of gamma and delta sites became evident only when the promoter strength was seriously reduced. The activity of the L30 promoter significantly decreased when three pyrimidine residues, spanning the transcription initiation site were converted to purines. It proves that the integrity of the oligopyrimidine tract is also a determinant of the transcription efficiency.

This information may provide a useful paradigm for understanding the transcriptional regulation of ubiquitously expressed genes.

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TARGETED CHANGES OF EMBRYONAL CELL DIFFERENTIATION IN A SERUM-FREE MODEL SYSTEM

Tu-141F.Bulić-Jakuš, V.Crnek, M.Vlahović and
G.Jurić-Lekić^aDepartment of Biology, ^bDepartment of Histology and Embryology, Medical Faculty, 41000 Zagreb, Šalata 3, Croatia

The most important aim of serum-free culture is to single out factors responsible for various cell functions. To investigate differentiation in gastrulating mammalian embryo, we developed an original serum-free organ culture. After two weeks on a metal grid, embryo forms a teratoma-like structure. As basic culture medium Eagle's Minimal Essential Medium (MEM) is used. In cultures with 50% rat serum (controls), epidermis, neuroblasts, columnar gut epithelium, cartilage and myotubes develop.

In rat embryos cultivated in pure MEM, keratinized epidermis and cartilage develop as in serum-supplemented medium. Changes of differentiation in serumless medium are obtained with: transferrin, albumin and retinoic acid (RA). The cells of the ocular lens differentiate in serumless medium with human transferrin, but not in MEM or other supplemented media. RA changes differentiation in MEM by inhibiting keratinization of epidermis which is then replaced with columnar gut epithelium (mucous metaplasia). As cartilage is also inhibited, the explants consist of gut epithelium, only. Bovine albumin in MEM promotes differentiation of all tissues as in serum-supplemented medium.

In conclusion we can say that our serum-free model system is a good test system to discover specific permissive signals for differentiation in mammalian embryo.

THE ROLE OF 3'-LYING SEQUENCES IN THE REGULATION OF H-2K_b GENE EXPRESSION**Tu-143**J.HATINA^{a,b}, P.JANSA^a, J.FOREJT^a and
L.REISCHIG^c^aInstitute of Molecular Genetics, CzAcadSci, Prague; ^bInstitute of Animal Physiology and Genetics, CzAcadSci, Liběchov;^cFaculty of Medicine, Charles University, Plzeň

Expression response of H-2K_b gene variants stably transfected into mouse L(tk-) fibroblasts, to IFNs and TNF- α has been studied. The cis-responsive elements have been mapped within the previously identified "downstream regulatory element" sequence, computational analysis of this sequence suggests several potential cis-regulatory sequences including ISRE, NF κ B and NFIL-6 binding sites.

A set of H-2K_b transgenic mice has been produced and tissue specificity and copy-number dependence of transgene's expression have been analysed. No correlation between number of copies and expression levels has been observed in various tissues (liver, kidney, lung), suggesting that an important regulatory element, probably on 3' end, is missing in the transgene. Correlation have been observed in lymphoid tissue, suggesting the existence of a different regulatory schema.

THE FIBRINOLYTIC AND TROMBOLYTIC PROTEIN FRACTION FROM TISSUE CELL CULTURE.

M.Leikina (a), V.Pastorova (b),
L.Lyapina (b), G.Leikina (a)

a. Department of Cytology and Histology; b. Laboratory of Blood Coagulation Biological Faculty, Moscow, 119899, Russia

As a subjects of investigation PKEV (pig, kidney, embryo, versen) monolayer culture will be used. This culture was made in 1957 by Kulikova in the Institut of poliomyelitis. This culture is enough stable, homogenous. The cell are polygonal, well spread.

The protein fraction obtaining the plasminogen activator activity has been isolated and purified from the all of the 9-12 day age culture by the gel-filtration method. The isolated fraction has not anti- and coagulating agents. The plasminogen activator activity of this fraction was 76,4U/mg.

The significante antithrombotic effects of the fraction was established: in albino rats the intravenous injection of tissue thromboplastin after the infusion of 38,2 U/200g plasminogen activator caused the death only at 20% animals by the vessel thrombosis whereas in control rat group it caused the death of 80% animals. The intravenous injection of the plasminogen activator (38,2 U/200g) to albino rats having the fresh thrombi in v.jugularis caused its lysis during 1-1,5 h. On 2 h after the injection of plasminogen activator to rats having the thrombi in v. jugularis the increase of fibrinolytic activity in the blood plasma as compared with the control animals has been established.

Tu-144 Involvement of Ets-Related Proteins in Hormone Independent Mammary Cell-Specific Gene Expression

Thomas Welte¹, Patricia Jennewein¹, Katja Garimorth¹, Sonja Philipp¹, Carmen Huck², Andrew C.B. Catō², and Wolfgang Doppler¹, ¹Institute of Medical Chemistry and Biochemistry, Fritz Preglstr.3, A-6020 Innsbruck, AUSTRIA.²Kernforschungszentrum Karlsruhe, Institute for Genetics, Postfach 3640, D-76021 Karlsruhe, GERMANY.

Regulatory regions have been located in the 5' flanking sequence of the mouse whey acidic protein (WAP) gene which contribute to its tissue and stage specific expression in the mammary gland. They can be functionally separated into elements which mediate the action of lactogenic hormones prolactin and glucocorticoids and elements which control mammary cell specific transcription in the absence of hormones. By mutational analysis, we have located a site in the WAP promoter between -120 and -100 which is important for hormone independent promoter function. In stably transfected HC11 mammary epithelial cells, the hormone independent activity of the mutated promoter was reduced 40-fold, whereas the capability to respond to lactogenic hormones was retained. The site was specifically recognised by two nuclear factors contained in extracts of cultivated mammary epithelial cells or mammary glands. Electrophoretic mobility shift assay, DNase I footprinting and methylation interference experiments indicated a relation of both factors to the ETS-family of DNA-binding proteins. One of these factors also recognised a functionally important site in the mammary cell specific enhancer of the mouse mammary tumor virus long terminal repeat. The results suggest that factors related to the ETS-family are important determinants in mammary cell specific gene expression.

**A New Member of the Ets-Family of Transcription
Tu-145 Factors Isolated from a Mammary Gland Library**

Judith Lechner, Thomas Welte and Wolfgang Doppler
Institut für Medizinische Chemie und Biochemie, Fritz-Pregl-Str. 3,
A-6020 Innsbruck, Austria.

The ETS-family of proteins is characterized by a conserved DNA-binding domain, which recognizes purine-rich target sequences. Members of this family have been implicated in the transcriptional regulation of genes specifically expressed in hematopoietic cells. We have recently identified a binding site for ETS-proteins in the promoter region of the whey acidic protein gene, which was found to be important for mammary cell specific expression. In an attempt to identify the ETS-proteins involved in that regulation, we isolated a novel member of the ETS-family from a mouse mammary gland cDNA library, using an approach employing degenerated PCR-primers located in the ETS-DNA binding domain.

The obtained sequence predicts a protein of 231 amino acids with a molecular mass of 26900 D. It belongs to the PEA3-subfamily and is most similar to ER81.

The protein was expressed in COS-7 cells and its DNA-binding properties in relation to other ets-proteins were investigated by electromobility shift assays.

**SEX DIFFERENCES IN PROLIFERATION OF RAT
AORTIC SMOOTH MUSCLE CELLS IN SHORT AND
LONG-TERM CULTURES**

Tu-146 L. Bačáková, F. Kocourek, V. Lisá, V. Mareš
**Institute of Physiology, Academy of Sciences of the Czech
Republic, Prague**

Activation of growth of vascular smooth muscle cells (SMC) is supposed to play an important role in the development of vascular diseases, mainly atherosclerosis and hypertension. In experimental animals and also in humans, some vascular disorders prevail, or are more severely expressed, in males.

In this study we report sex differences in proliferation in aortic SMC prepared from young adult SPF Wistar rats. The explants of the aortic tunica media were cultured in modified Eagle minimum essential medium with fetal calf serum (10%) and gentamycin (40 µg/ml). The samples from each animal were cultured separately or pooled from 4 animals for each sex.

In the 2nd passage cultures at the early exponential phase of growth (day 1 to 4 after seeding), the doubling time was not found different in both sex group (35.8±3.9h in males and 41.9±12.5h in females); there were, however, great inter-individual differences within each sex group. In the late exponential phase of growth (day 4 to 7), the doubling time was significantly shorter in males (69.7±5.6h vs. 207.7±52.3h in females, p<0.05). By the end of the experiment on day 7, the cell population density in female derived cultures reached its maximum (40565±14348 cells/cm²) while in the male derived cultures it reached 57704±7884 cells/cm² and it continued growing. Similar results were obtained in cultures prepared from samples containing material pooled from 4 donors of the same sex. The sex differences in the studied SMC proliferation parameters persisted for 21 passages (i.e. for about 9 months in vitro). Therefore, the data revealed significant sex differences in growth potential of the aortic SMC in cultures and suggest a long-term fixation of the differences in expression of genes taking part in regulation of SMC growth in both sexes.

**A RELIABLE MATCHING ALGORITHM FOR
QUANTITATIVE GEL ELECTROPHORESIS
Tu-148 ANALYSIS**

Jiří Vohradský
**Czech Academy of Sciences, Inst. of Microbiology,
Praha, Czech Republic**

Correct alignment of bands or spots among set of one-dimensional (1-D) or two-dimensional (2-D) gel electrophoretograms is of basic importance in quantitative interpretation of electrophoretograms. Due to the local inhomogeneities in electrophoretic process, gel matrices, gel manipulation, sample differences etc., direct alignment is rarely possible. Matching algorithm must be able to correctly pair corresponding bands or spots among a set of different gels in a reasonable time with very high accuracy. We have developed reliable matching algorithm based on optimisation of mutual local correlation between two gels, by minimising defined objective function. The algorithm generates a "displacement" function allowing local transformation of compared gel. Final matching is accomplished by examining close neighbourhood of a band in reference and transformed gel. The discussion of the algorithm, objective function and the "displacement" function will be given. The performance analysis of the algorithm will be given using several examples.

CANCELLED

GENE TARGETING IN EPITHELIAL CELLS OF
THE ENDODERMAL CELL LINEAGE USING
THE HUMAN VILLIN PROMOTER.

Tu-149

S. Robine^a, E. Caliot^a, D. Pinto^a, J.J. Fontaine^a, G. Trempe^b, M. Buchwald^c, Ch. Babine^a, D. Louvard^a
and E. Pringault^a

^a Institut Pasteur, 25 rue du Dr. Roux F-75015 Paris, France. ^b Rhône-Poulenc Rorer, F-94403 Vitry-sur-Seine, France. ^c Hopital for Sick Children, Toronto, Canada.

The 2 kb 5'-flanking region of the human villin gene has been demonstrated to contain sufficient regulatory elements to recapitulate the expression pattern of the villin promoter during intestinal differentiation (Robine et al., 1993, *J.Biol.Chem.*, 268:11426-11434).

The ability of this villin promoter to target heterologous gene in vivo in the endodermal cell lineages was checked in transgenic mice. We have obtained transgenic mice harboring the activated Ha-ras oncogene under the control of the villin gene regulatory sequences. The expression of the activated Ha-ras is restricted to small and large intestine and epithelial cells from the thymus (which contains a subpopulation of villin positive cells). The physio-pathological consequences are analysed.

In order to generate new digestive epithelial cell lines in culture, we have obtained transgenic mice expressing the thermo-sensitive allele of the SV 40 large T antigen under the control of the villin promoter.

We also succeeded to target the expression of the human CFTR gene driven by the same regulatory sequence of the human villin gene, in the intestinal crypts of transgenic mice.

CANCELLED

INCOMPLETE ACTIVATION OF SEA URCHIN
EGGS UNDER THE INFLUENCE OF
DIMETHYLSULFOXIDE

Tu-152

E.Gakhova, N.Chekurova

Institute of Cell Biophysics RAS, Pushchino,
Moscow Region, Russia

Fertilization membrane was induced in dimethylsulfoxide (DMSO) treated eggs of the sea urchin *Strongylocentrotus intermedius*. The number of the eggs with fertilization membrane was dependent on DMSO concentration in the range of 0,5-5 M. DMSO at above 3 M activated almost all eggs. The formation of the fertilization membrane was inhibited in Ca²⁺-free artificial sea water with 50 mM EGTA or in the presence Ca-channel blockers Co²⁺, Cd²⁺, Mn²⁺ at 10⁻⁴ M. These data indicate that Ca²⁺ plays a predominant role in the DMSO-induced formation of the fertilization membrane.

Ultrastructural changes in the egg membranes induced by DMSO were very similar to those found in the process of insemination or parthenogenetic activation of the egg. However, cleavage of the eggs treated by DMSO did not occur.

CANCELLED

Tu-153 MITOTIC FRAGMENTATION OF THE GOLGI COMPLEX IN A CELL-FREE SYSTEM

Tom Misteli and Graham Warren
Imperial Cancer Research Fund, Cell Biology Laboratory, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.

During mitosis, the Golgi complex of mammalian cells disassembles into clusters of 50–70nm uncoated vesicles, which disperse throughout the cytoplasm during anaphase. We have developed a cell-free system to study this process morphologically at high resolution as well as biochemically. When purified rat liver Golgi membranes were incubated with mitotic cytosol from prometaphase arrested HeLa cells, the membranes lost their typical stacked appearance and fragmented into uncoated vesicles and tubules. This morphological change was dependent on mitotic cytosol, energy in the form of ATP, elevated temperature, and the action of the mitotic master regulator cdc2 kinase. By applying stereological methods we showed that the production of coatomer-coated intra-Golgi transport vesicles from Golgi cisternae was not affected by mitotic cytosol, neither was the rate of coat-shedding. When a component of the coatomer was depleted from mitotic cytosol the fragmentation process did not take place, but extensive tubular networks were observed. This data supports a model in which continued budding of coatomer-coated transport vesicles with an inhibition of their fusion with their target membranes contributes to the fragmentation of the Golgi complex during mitosis.

Tu-154 ACTIVITIES OF GOLGI APPARATUS IN LEAVES DURING THE EFFECT OF ETHEPHON

M. Selga & T. Selga
Institute of Biology, Academy of Sciences of Latvia 3 Miera st., Salaspils LV 2169, Latvia

The ultrastructure of Golgi apparatus was studied in relation with plant growth regulation. Rye (*Secale cereale L.*), cucumber (*Cucumis sativus L.*) and pea (*Pisum sativum L.*) plants were sprinkled with a retardant Ethepron in a concentration of 4×10^{-2} M. It causes inhibition of plant growth, promotes development of side shoots and appearance of a great amount of Golgi stacks in the palisade parenchyma cells of mature leaves of the basic shoot. They were diverse and more than 50 types with different position and configuration were observed: in the diverse relations with ER, near the plasmalemma, tonoplast, nuclei, chloroplasts, mitochondria, or in complex chains of some organelles. The Golgi stacks were turned towards them both with the convex (forming), concave (maturing) face or sideways. It appears that Ethepron causes in the same cells at the same time several activities of Golgi apparatus: increased production and transport of proteins, polysaccharides and possibly conjugates of ethylene to the periphery of cytoplasm, into the vacuoles, in the secretory pathway; a two way interaction with the DNA containing organelles by exchange of substances/energy and in changing of the organelle cycle; the formation/thickening of the cell envelope, plant detoxication and endogenous regulation.

Tu-155 DISSECTION OF ENDOCYTIC COMPARTMENTS USING IODIXANOL DENSITY GRADIENTS

J. Graham and D. Billington
School of Molecular Sciences,
Liverpool John Moores University, Liverpool, UK

Iodixanol is a new low viscosity, iso-osmotic centrifugation medium which can form useful self-generated gradients in fixed-angle, near-vertical and vertical rotors at 250,000g for 1h. We have used it to fractionate the endocytic compartment of rat liver: for buoyant density separations the homogenate or post-nuclear supernatant is simply adjusted to 12.5% Iodixanol and then centrifuged; for rate-zonal separations the sample is placed on top of a self-generated gradient and then centrifuged at 40,000g for 40 min. These conditions provide a shallow gradient in which to resolve the endosome populations and allow major organelles (lysosomes, endoplasmic reticulum, mitochondria etc) to band close to the wall of the tube.

99m Tc-labelled neo-galactosyl albumin (NGA - a synthetic ligand to the asialoglycoprotein receptor) is rapidly endocytosed by the perfused rat liver: within 1 h, 40% of the internalised ligand is expressed into the bile, 80% of which is in a degraded form. Analysis on Iodixanol gradients was performed at 1, 2, 10, 20 and 40 min after a 1 min pulse of NGA.

Between 1 and 10 min, NGA moves from a dense endosome (1.095 g/ml) to light endosome (1.075 g/ml) and at 2 min at least two endosome populations can be separated on the basis of size. Between 10 and 20 min the label shifts to the lysosomes and into another fraction which may contain the bile canalculus: this is currently under investigation.

Tu-156 3-METHYLADENINE INHIBITS TRANSPORT FROM LATE ENDOSOMES TO LYSOSOMES IN CULTURED FIBROBLASTS

E.-L. Punnonen* and H. Reunanan

Department of Biology, University of Jyväskylä, Finland
*Present address: Abteilung Biochemie 2, Georg-August Universität, Göttingen, Germany

The effect of 3-methyladenine on transport from endosomes to lysosomes was studied in rat embryonic and mouse 3T3 fibroblasts. Subcellular fractionation in 27% Percoll gradients showed that pre-endocytosed (5 min pulse) horseradish peroxidase (HRP) was not transported from endosomes to dense lysosomes in cells chased in the presence of 10 mM 3-methyladenine. However, fractionation in 20% Percoll gradients, which separated early endosomes from late endosomes and lysosomes, as well as light and electron microscopical experiments, showed that HRP was transported from early endosomes to the perinuclear late endosomes.

Immunoprecipitation of metabolically labelled cells was used to study the biosynthetic processing of a lysosomal proteinase, cathepsin L. The results showed that the early processing of the precursor to the intermediate form was not affected by 3-methyladenine, while the late processing of the intermediate to the mature form was retarded. The early processing begins in a post-Golgi compartment, i.e. late endosomes, and the late processing is completed in mature lysosomes. Immunofluorescence labelling showed that, after 2 h of treatment in 3-methyladenine, cathepsin L had accumulated in the perinuclear area. Another lysosomal enzyme, β -glucuronidase, was normally distributed in both perinuclear and peripheral vesicles which indicated that the localization of lysosomes was not altered. The results thus suggest that the late processing of cathepsin L was inhibited because transport from perinuclear endosomes to lysosomes was retarded.

In conclusion, both endocytic pulse-chase experiments and immunoprecipitation of metabolically labelled cells suggest that 3-methyladenine inhibits transport from late endosomes to mature lysosomes in both rat and mouse fibroblasts.

LYSOSOMAL ALTERATIONS INDUCED BY AZITHROMYCIN (Az) IN CULTURED RAT EMBRYO FIBROBLASTS
 J. Piret, C. Gerbaux, J.-P. Montenez and P.M.
Tu-157 Tulkens
 Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain & ICP, Avenue Hippocrate, 75 Bte 75.39, B-1200 Bruxelles, Belgium

Azithromycin, a recent acid-stable macrolide antibiotic, demonstrates an exceptional accumulation in lysosomes of cultured cells. Electron microscopic examination of rat embryo fibroblasts incubated with 10 mg/l of drug for 24h, revealed that this huge accumulation is associated with the development of alterations in this organelle, i.e. lysosomes become enlarged and overloaded with osmophilic, lamellar structures (pseudomyelinic figures), together with electron-dense and heterogeneous material. Biochemical analysis indicated that the total lipid phosphorus content of these cells was markedly increased in a time- and a dose-dependent manner. Because Az appeared to modify the catabolism of lipids, and perhaps other substances in the lysosomes, we investigated its influence on the activities of several enzymes at increasing drug concentrations.

Az Conc.	10 mg/l	50 mg/l	250 mg/l
Pase A1(1)	105.3±5.3	125.2±29.0	55.3±3.3
	139.6±21.4	113.6±16.7	38.0±17.4
SMase	79.2±10.4	34.3±5.3	3.5±0.7
Cath. B	175.9±30.8	87.4±11.7	6.9±0.2

Pase A1 = phospholipase A1 activity (1) towards phosphatidylcholine, (2) towards phosphatidylethanolamine, SMase = sphingomyelinase and Cath. B = cathepsin B; Activities are expressed in % of control without drug added

Thus, at 10 mg/l Az, concentration for which conspicuous morphological alterations of lysosomes are seen, only the activity of sphingomyelinase was modestly decreased whereas that of the other enzymes tested were significantly increased. At the highest dose, however, the activities of all enzymes were markedly decreased. This suggests that the lysosomal overloading induced by Az may not result from a primary effect of the drug on the enzymes, but from an hitherto undescribed indirect effect.

Intracellular sorting of M_r 46 kDa mannose 6-phosphate receptor (MPR 46)

Tu-159 C. Schulze-Garg, C. Böker, E.-L. Punnonen, K. von Figura, and A. Hille-Rehfeld

Biochemie II, Universität Göttingen, D-37073 Göttingen, Germany

Mannose 6-phosphate-specific receptors recycle between *trans* Golgi network and endosomes for transport of newly synthesized lysosomal enzymes. To search for signals that mediate sorting of MPR 46, peptide-specific antibodies that bind to the cytoplasmic domain of MPR 46 have been injected into cultured human fibroblasts to interfere with intracellular transport of the receptor. Binding of antibodies to peptide 43-47 (Ala-Tyr-Arg-Gly-Val) efficiently blocked return of MPR 46 to the *trans* Golgi network. Antibody-induced redistribution caused accumulation of MPR 46 within an endosomal subcompartment, which was accessible to endocytosed antibodies against the ectodomain of MPR 46, but which apparently is not passed by endocytosed material en route to lysosomes (Schulze-Garg et al. 1993. J. Cell Biol. 122:541-551).

According to the pH- and ligand-dependent equilibrium for oligomerization and dissociation of subunits of MPR 46 *in vitro*, it has been postulated that recycling of MPR 46 between the *trans* Golgi network and endosomes is accompanied by changes in its quaternary structure (Waheed and von Figura 1993. Eur. J. Biochem. 193:47-54). This issue is currently investigated using monoclonal antibodies, which preferentially recognize oligomeric forms of MPR 46. A monoclonal antibody which preferentially recognizes tetrameric over dimeric or monomeric MPR 46 was shown to label a subset of MPR 46-containing membranes in immunofluorescence. This monoclonal antibody labelled the endosomal subcompartment of redistributed MPR 46 very efficiently, suggesting that it contains, at least partly, tetrameric forms of MPR 46.

HOW CELLS ACQUIRE IRON IN PROTEIN - FREE MEDIUM

Tu-158 K.Kriegerbecková^a, B.Scheiber^b, J.Kovář^a and H.Goldenberg^b

^aInst. of Molecular Genetics, Academy of Sciences, Prague; ^bInst. of Medical Chemistry, University of Vienna.

Cells cultured in a protein-free medium must be supplied with iron in a soluble complex to be able to grow. We have adapted cell lines which can grow in medium with very low ferric citrate concentration (<5μM). This property is confined to few cell lines, while practically every type of cultured cells grows in media with high ferric citrate concentration (500μM). The "low-iron dependent" cells do not produce any transferrin to make the iron available to them.

Adapted HeLa cells can take up iron from iron-citrate complexes of molecular stoichiometry of 1:1.5. The rate is equal to that of uptake from a Fe-DTPA (1:4) complex. It is not inhibited by metabolic inhibitors, divalent metal ions (cooper, cobalt, manganese) or hexacyanoferrates (II as well as III) and is nearly temperature independent. Between pH 6 and 8 the uptake rate is unchanged and only rises at lower pH. It is blocked by a surplus of the strong ferric chelator DTPA, but only weakly inhibited by a 1:1 Fe-DTPA chelate. A surplus of iron-citrate complex has practically no influence on this uptake mechanism. Cells grown in "high-iron" medium (500μM Fe-citrate), display practically the same characteristics of iron uptake. In contrast to cells grown in a medium containing transferrin cells grown in low-molecular weight iron have much higher intracellular iron.

We conclude that unspecific pinocytosis is the mechanism of uptake from the citrate complex, in contrast to that from Fe-DTPA, and is completely sufficient for an adequate iron supply from a medium with low iron concentration in cells adapted to these cultured conditon.

BIOCHEMICAL AND ULTRASTRUCTURAL CHANGES IN BAL -

ISOLATED CELLS AFTER BCG - VACCINE ACTIVATION IN EXPERIMENTAL LUNG EMPHYSEMA

Sulkowski S.^a, Chyczewski L.^a, Skrzylewska E.^b, Sulkowska M.^a, Worowski K.^b

^a Department of Pathological Anatomy and ^b Instrumental Analysis, Białystok Medical School, Poland

Our previous studies showed that BCG - vaccine given s.c. or i.p. stimulates the rats pulmonary alveolar macrophages (Chyczewski L., Sulkowski S., Exp Pathol. 1988;34:41-50.). That resulted in the enhancing of papain - induced lung emphysema. Aim of the present study was to evaluate Cathepsin D, base proteases, antiproteases, antitrypsins, antichymotrypsins and protein content in the 24 h culture medium of the BAL - isolated cells deriving from the rats stimulated with BCG - vaccine and papain - induced emphysema. Morphological and morphometrical analysis of the lungs and ultrastructural (SEM) examinations of isolated cells were performed too. The experiment was carried out on male Wistar rats, of 180 - 220 g b. w. Two i. p. injections of BCG - vaccine (4×10^8 microorganisms) on the 1st and 14th days were applied as macrophages mobilizing and activating agent. Papain (2 mg / 1ml / 100 g b. w.) was given once i. t. on 21st day. The animals were sacrificed on 28 th day of experiment.

An ultrastructural changes suggested increased activity of cells isolated from BCG-treated rats. In the culture medium of cells isolated from the rats which were given BCG or papain and BCG + papain we observed increased of base proteases activity and decreased of Cathepsin D activity comparing with control group. Increased antitrypsin activity in the BCG- and BCG + papain - treated rats and decreased antitrypsin activity in papain - treated rats only was observed too. There was not obvious differences in the levels of the antiproteases and antichymotrypsins activities between examined groups. In the lungs of animals treated with BCG + papain we observed morphometrically enhancing of emphysematos changes comparing with the papain - treated rats only.

The obtained results indicate that activated pulmonary macrophages are one of the sources of the protease-antiprotease intracellular imbalance. However increased production of proteolytic enzymes may not be the only factor responsible for the progression of lung emphysema in BCG-treated rats. Another factors derived from pulmonary macrophage (e.g. cytokines) must be considered.

MELANOGENESIS IN TRANSFECTED FIBROBLASTS INDUCES LYSOSOMAL ACTIVATION

Tu-161 J. Borovansky, D. Egendaal, N. Smit, M. Mommaas, B.J. Vermeer and S. Pavel

Department of Dermatology, University Hospital, Leiden, The Netherlands

Melanin production (melanogenesis) is the best defined differentiated function of pigment cells. As in this process cytotoxic species are produced melanogenesis is compartmentalized to melanosomes. Melanosome biogenesis requires fusion of structural proteins with tyrosinase. 3T3 Swiss mouse fibroblasts transfected with mouse tyrosinase cDNA -clone 1.13.4 (Winder et al. J Cell Sci 104, 1993, 467) represent a unique system in which melanogenesis takes place in the absence of structural proteins.

Transfected fibroblasts displayed tyrosinase activity and produced pigment granules. In the absence of structural proteins the granules failed both to have a typical ultrastructure and to pass the usual melanosome ontogenesis. Differentiating agents like DMso increased pheomelanin production. Pigment was localized within membrane-bound vesicles resembling lysosomes. Clone 1.13.4 had a significantly higher level of lysosomal enzymes (β -hexosaminidase, α -mannosidase) compared both to 3T3 cells and to clone PKG4 (fibroblasts transfected with the G410 resistance plasmid). Melanosomal proteins can act as scavengers of toxic products of melanogenesis. Our data suggest that in their absence cells may employ an alternative way to sequester injurious products and seem to explain the activation of lysosomal apparatus observed in some pigmentation-associated mutations.

UPTAKE OF IRON FROM A DTPA-CHELATE BY ISOLATED RAT HEPATOCYTES

Tu-163 B.Scheiber and H.Goldenberg

Inst. Medical Chemistry, University of Vienna,Austria

Uptake of iron by hepatocytes from Fe-DTPA was saturable and inhibitable by a surplus of a 1:1 Fe-DTPA complex. The rates were higher than those obtained with diferric transferrin as iron source. The uptake was suppressed by pretreatment of the cells with trypsin and totally or partially inhibited by transition metal ions (copper, manganese, cobalt, zinc), azide, NEM, chloroquine, baflomycin and methylamine, but not monensin. The pH-optimum was 6.0. Higher concentrations of free DTPA inhibited iron uptake as well as polymer-conjugated desferrioxamine. Apo-transferrin showed a very similar effect. Neither transferrin nor the polymer-bound DFO were able to bind iron from the DTPA-complex by themselves. DTPA as well as polymeric DFO also inhibited iron uptake from diferric transferrin. The hydrophilic BPS stimulated iron uptake from the Fe-DTPA complex, while the hydrophobic dipyridyl inhibited at the same concentration. Uptake was also stimulated by ascorbate and by dehydroascorbic acid.

After uptake at 16°C and removal of surface-bound iron, the iron was found at a density of approximately 1.1g/cm³ in a sucrose density gradient. On attempting to pulse-chase this label at 37°C, 75% were lost to the chase medium, while the rest now was cytosolic as seen after uptake at 37°C.

Treatment of the homogenate of iron-loaded hepatocytes with graded concentrations of ammonium sulfate in the cold almost completely precipitated the radioactive label. No membrane or other proteins were radioactively labelled by the iron except ferritin at any uptake temperature.

MEMBRANE ASSOCIATION OF TWO CONCANAVALIN A-DIFFERENTIATED α -ACID GLYCOPROTEIN GLYCOFORMS IN THE GOLGI COMPLEX OF ISOLATED RAT HEPATOCYTES

C. POÙS, A. DRECHOU and G. DURAND

Laboratoire de Biochimie Générale, Faculté de Pharmacie, 5, Rue J.B. Clément, CHATENAY-MALABRY, FRANCE

We have shown previously that two Concanavalin A-differentiated glycoforms of various glycoproteins, including α -acid glycoprotein (AGP), are secreted at different rates by isolated rat hepatocytes, the nonretained (NR) form being secreted faster than the retained (R) form [Biochem. J. (1989) 263, 961-964; Eur. J. Biochem. (1992) 203, 277-283]. This phenomenon occurs in the medial or trans-Golgi of hepatocytes, and we thus tested whether it involved an interaction of AGP with Golgi membranes.

Collagen-seeded hepatocytes pulse-labelled with [³⁵S] methionine for 10 min and chased for 45 min were permeabilized with 3 mg/ml saponin for 40 min in fresh chase medium. A small proportion of intracellular albumin was retained in hepatocytes (15.6 ± 1.3%) whereas significantly more AGP was retained (28.7 ± 3.5%). AGP immunoisolated from permeabilized hepatocytes was not deglycosylated by Endo-H treatment, confirming that retained AGP had already reached the medial Golgi. Two membrane fractions enriched mainly in medial and trans-Golgi were prepared by ultracentrifugation from 4h-labelled hepatocytes. Permeabilization by 0.5 mg/ml saponin led to 20.9 ± 4.6% and 67.0 ± 7.5% retention of albumin and AGP respectively in the membrane fraction. ConA-Sepharose analysis showed that membrane-associated AGP comprised 85.2 ± 7.0% of the R-AGP form and only 33.5 ± 10.5% of the NR-AGP form.

AGP, a soluble plasma protein, may thus undergo marked association with Golgi membranes during its secretion by hepatocytes. The major interaction of R-AGP with Golgi membranes could account for its slow secretion by isolated hepatocytes.

RELATIONSHIP BETWEEN LOCALIZATION OF SUBSTANCE IN THE MEMBRANE AND PROPERTIES OF THE MEMBRANE

Tu-164 A. Gawron^a, K. Wójtowicz^b, L.E. Misiak^c, W.I. Gruszecki^c

^a Department of Cell Biology, Maria Curie-Skłodowska University, Lublin, Poland; ^b Department of Biophysics, Medical Academy, Lublin, Poland; ^c Institute of Physics, Maria Curie-Skłodowska University, Lublin, Poland

Lutein and 8-methoxysoralen (8-MOP) are the compounds which chemical structures and pharmacological activity are very different. Lutein (polar carotenoid) is located across the membrane. Localization of 8-MOP with respect to lipid bilayer is similar to that of cholesterol. For these reason we compared the effect of lutein and 8-MOP in the dark on liposomal and erythrocyte membranes by means of a spin label, ultrasound absorption and calorimetric techniques.

It was shown that incorporation of 5 mol% lutein or 8-MOP into erythrocyte membranes or dipalmitoylphosphatidylcholine (DPPC) liposomes increases fluidity of the membrane in the lipid core adjacent to the hydrophilic zone (as monitored with 5-doxyl stearic acid spin label). Both compounds increased evidently ultrasound absorption in the DPPC liposomes without affecting drastically the phase transition cooperativity. Cooperativity of the main phase transition of DPPC liposomes was slightly decreased in the presence of 8-MOP and clearly decreased in the presence of the same amount of lutein. In both cases the phase transition was shifted towards lower temperatures in the presence of additives.

This results indicate that mode of action these substances on membranes was very similar and consist in fluidizing of the hydrophobic core of the membrane. However degree observed changes was much better for located across the membrane of lutein than situated in the lipid core adjacent to the hydrophilic zone 8-MOP.

Tu-165 IDENTIFICATION OF CYCLIN A IN RAT LIVER ENDOCYTIC FRACTIONS. EXPRESSION DURING LIVER REGENERATION.

Marcel Vergés, Anna Castro, Oriol Bachs and Carlos Enrich.

Dept. Cell Biology. Faculty of Medicine. University of Barcelona. Spain.

We have identified cyclin A in the different fractions of endosomes isolated from rat liver. Cyclin A was mainly located in the late endocytic fraction and by immunocytochemistry showed an intense staining in the peri-canicular region of hepatocytes. The expression, by Western blotting, of cyclin A during the pre-replicative phase of liver regeneration showed a significant increase with a peak around 12 hours after a partial hepatectomy. In addition, its specific kinase cdc2 was present in the endosomes from 18, 24, 36 and 48 hours whereas the cdk2 was present in control and during the G1 phase (0 to 18 hours). These results indicate that the arrest of intracellular trafficking during the mitosis is specifically modulated at the endocytic compartment.

Tu-167 Interactions of the 300 kDa mannose 6-phosphate receptor with cytosolic and membrane-associated proteins

O. Rosarius, K. von Figura and T. Bräulke
University of Göttingen, Department of Biochemistry II,
D-37037 Göttingen, Germany

The 300 kDa mannose 6-phosphate receptor (MPR300) mediates the transport of mannose 6-phosphate containing ligands from the trans-Golgi network and the cell surface to the endosomal compartment. The cytoplasmic tail of the human MPR300 (MPR300-CT) is phosphorylated at 4 to 5 serine residues. To investigate the role of MPR300 phosphorylation the phosphorylation sites and the kinases were examined. The entire coding sequence of the MPR300-CT was expressed in *E. coli* and following isolation used as a substrate for different purified kinases. Inhibition studies using synthetic peptides, partial sequencing of isolated tryptic phosphopeptides and co-migration with tryptic phosphopeptides from MPR300 phosphorylated *in vivo* showed that casein kinase II (CKII) phosphorylates the MPR300-CT *in vitro* and *in vivo* at serine residues 82 and 157. Proteinkinase A (PKA) phosphorylates the MPR300-CT only *in vitro* at serine residue 20 and a non identified site. Cross-linkage studies showed that the CKII phosphorylated MPR300-CT interacts mainly with a cytosolic protein of 35 kDa (TIP35) and with 35 kDa and 91 kDa proteins removed from rat brain membranes by high salt washing. Gel filtration suggests that both 35 kDa proteins are part of a higher molecular mass complex of approx. 130-150 kDa. Inhibition studies using phosphorylated and non-phosphorylated MPR300-CT indicate that the interaction with TIP35 is dependent on phosphorylation. Furthermore, TIP35 was only cross-linked to the MPR300-CT phosphorylated by CKII but not to MPR300-CT phosphorylated by PKA. This interaction is increased by divalent cations and inhibited by low concentrations of ATP. The salt-washed 35 kDa and 91 kDa membrane proteins interact with the MPR300-CT in a phosphorylation-independent manner. The results suggest that cytosolic and membrane associated proteins may interact with different structural determinants in the cytoplasmic domain of MPR300 which include serine residues phosphorylated by CKII.

Tu-166 REACTION OF CELL MEMBRANES AND GROUND CYTOPLASM TO TREATMENT WITH XANTHOTOXIN

A. Gawron^a, C. Ashby^b and A.M. Zobel^b

^aBiology Dept., Lublin University, 20-007 Lublin, Poland; ^bDept. of Chemistry, Trent University, Peterborough, ON, Canada K9J 7B8

Xanthotoxin is used clinically as an anticancer drug, but not much has been done on the biochemical mechanism of its reaction and on changes it causes to the membranes. We found that xanthotoxin altered plasma membrane permeability and caused segregations of some areas of cytoplasm. Large areas of segregated cytoplasm, surrounded by endoplasmic reticulum membranes, were either more or less electron-dense than the surrounding ground cytoplasm, the latter caused by disappearance of ribosomes followed by degradation of cytoplasm. Many vesicles contained increased amounts of lysosomal enzymes, which were found as well in higher concentrations outside the cells.

Biochemical investigations showed that xanthotoxin retarded oxidative phosphorylation and lowered oxygen consumption. Chromosomal aberrations were observed during mitosis. From the above observations we conclude that use of xanthotoxin as a safe anticancer drug is problematical.

CAVEOLAE SEEM TO BE DISTINCTIVE STRUCTURES IN ELICITATED MACROPHAGES

Tu-168 Anna L.Kiss, Lab.I.of Electron Microscopy Semmelweis University of Medicine, H-1450 Budapest, Hungary

Caveolae are flask- or omega-shaped plasma membrane invaginations presenting in numerous cell types. They must represent functionally important organelles, but whether caveolae in various cells have the same function is not known. There are no data available whether caveolae are present in macrophages as well.

The aim of our study was to identify caveolae (or caveolae-like structures) in resident and elicited macrophages and to try to obtain some evidence about their possible function.

We have studied fluid phase (HRPO uptake) and receptor-mediated endocytosis (internalization of PAP immune complex) in resident and elicited macrophages. The amount of the internalized HRPO and PAP was measured spectrophotometrically. We have also done morphometrical analysis on empty coated vesicles, caveolae and HRPO- or PAP-labelled structures in both cells.

Our results show:

- 1.) Caveolae-like structures can be found in significantly larger number in elicited macrophages than in resident cells. This result suggests that the appearance of caveolae-like structures on the cell surface can be a highly regulated process and the number of these invaginations can vary with the functional activity of the cells.
- 2.) The diameter of these profiles was found around 90 nm in elicited and 70 nm in resident macrophages.
- 3.) Caveolae can take part in fluid phase and receptor-mediated endocytosis in elicited macrophages.

LIPID TRAFFIC IN EPITHELIAL CELLS

Tu-169 Ida van Genderen and Gerrit van Meer
Department of Cell Biology, Medical School,
University of Utrecht, the Netherlands

The apical plasmamembrane domain of epithelial cells is enriched in glycolipids, especially glucosylceramide. Sorting occurs after synthesis, before delivery at the plasma membrane domains. Additional sorting in the transcytotic pathway is required to maintain the generated polarity, as considerable amounts of membrane are transported to and from the opposite domain. We tested this by inserting short-chain NBD analogs of an 'apical' lipid, glucosylceramide, and a 'basolateral' lipid, sphingomyelin or galactosylceramide in either the apical or the basolateral membrane domain of different cell types (MDCK I,II Caco-2 and BeWo) and then allowing transcytosis. Unexpectedly no sorting could be detected. We do not know yet whether this means that there is no sorting in the transcytotic route, or that the short-chain NBD analogs are not recognized by the sorting machinery.

We found that these lipid probes actually followed predicted bulk membrane flow and used them as markers to obtain quantitative data on the kinetics of endocytotic and transcytotic membrane transport in different cell types. In MDCK II cells about 30% of each membrane surface was transcytosed per hour, while 2-3% of the glycolipid probes was degraded per hour. In MDCK I cells degradation was the same, 30% of inserted lipid was transported from apical to basolateral in one hour, while transcytosis from basolateral showed a lag time of 1-2 hours and was 10% per hour. In Caco-2 cells after apical uptake more lipid was degraded than transcytosed: 10% versus 6% per hour. From basolateral, 23% was transcytosed and 3% was degraded.

We will report on our current investigations on transport from early endosomes in different cell types, on the changes in kinetics with time in culture, drugs and other factors influencing vesicular transport.

Tu-171 CYTOCHEMICAL ANALYSIS OF DIFFERENT VACUOLES IN THE GALLBLADDER EPITHELIUM

M. Pšeničnik and N. Pipan

Institute of Cell Biology, Medical Faculty, Ljubljana 2, 61000 Ljubljana, Slovenia

The great variability in the morphologic appearance of the vacuolar structures in the mouse gallbladder epithelial cells was the basis for the cytochemical analysis of these compartments. The Golgi complex as the central part of the endomembranes system involved into the processing of cell products destined for the exocytosis and to lysosomal compartments was pointed out. Since the heterogeneous population of vesicular formations take part in several transport pathways the cytochemical markers for cis, medial and trans Golgi part, were used to get informations about the nature of different vacuoles structures. The activity of acid phosphatase was determined for the lysosomal identification, and the mucosubstances were recognised by tannic acid. After prolonged osmification Os-black is found constantly in the cis Golgi saccules. A number of cells contain Os black also in the segments of ER, perinuclear space and vacuoles with homogeneous or multi vesicular content. The reaction product of NADP-ase activity is not bound only to medial cisternae of Golgi complex but is prominent also in the majority of different vacuolar structures. The activity of acid phosphatase is found in particular homogeneous vacuoles while the reaction product of TPP-ase activity is exclusively in trans Golgi part. Small apical vesicles showing TA staining are secretory granules containing mucosubstances. These results suggest that the structures positive for NADP-ase and AP-ase can be regarded as lysosomes irrespective of their morphologic appearance. The reason why the content of particular vacuolar structures reduce OsO_4 to Os-black remains not cleared up.

ENDOSOME-LYSOSOME TRANSFER OF INSULIN AND ITS RECEPTOR IN A LIVER CELL-FREE SYSTEM.

Tu-170 G. Chauvet, B. Desbuquois, et K. Tahiri

INSERM U 30, Hôpital Necker Enfants-Malades, 75015 Paris, France

Upon interaction with liver cells, insulin is rapidly internalized along with its receptor into components of the endosomal apparatus. However, unlike other ligands, it is degraded at this early endocytic step and little associates with lysosomes. In the present study, the transfer of *in vivo* internalized insulin and its receptor from endosomes to lysosomes has been examined in a cell-free system. Livers were obtained from rats after iv injection of ^{125}I -labeled (0.05 nmol) or native insulin (10 nmol). Following homogenization, postmitochondrial supernatants were isolated, incubated at 37°C in the presence of ATP, and subfractionated in Nycodenz density gradients. In liver supernatants isolated after injection of ^{125}I -labeled insulin, the trichloroacetic acid-precipitable radioactivity was recovered at densities of 1.08-1.10, as was galactosyltransferase (a Golgi marker). Upon incubation, up to 30% of the radioactivity was shifted towards densities of 1.12-1.14, coinciding with acid phosphatase (a lysosomal marker). Transfer required at least 10 min incubation, was maximal 8 min after injection of radiolabeled insulin, required cytosol, and was unaffected by chloroquine (an acidotropic drug) and monensin (a proton ionophore). Following injection of native insulin, about 70% of insulin and 85% of insulin receptors in liver supernatants (as measured by radioimmunoassay and *in vitro* insulin binding, respectively) were recovered at densities of 1.08-1.10. Upon incubation, immunoreactive insulin was partially shifted towards higher densities but receptor distribution remained unchanged. SDS polyacrylamide gel electrophoresis analysis of supernatants isolated after injection of ^{125}I -labeled or native insulin showed that the α and β subunits of the insulin receptor (as detected by crosslinking and immunoblotting, respectively) were recovered mainly at the endosomal position. However, upon incubation, both subunits were partially shifted towards the lysosomal position. It is concluded that, in a liver cell-free system both internalized insulin and its receptor are partially transferred from endosomes to lysosomes, and that during this process the receptor loses its ability to bind insulin.

Tu-172 CYTOCHEMICAL DETECTION OF ENDOCYTIC COMPARTMENTS IN SUPERFICIAL CELLS OF MOUSE URINARY BLADDER

R. Romih^a, K. Jezernik^a, T.-T. Sun^b, B. Goud^c

^aInstitute of Cell Biology, Medical Faculty, LJUBLJANA, Slovenia; ^bEpithelial Biology Unit, Department of Dermatology, New York University Medical Center, NEW YORK, USA; ^cUnité de Génétique Somatique, Institut Pasteur, PARIS, France

In most cells endocytosis is a mechanism for internalization of extracellular materials and plasmalemma in which different endocytic compartments take part. In superficial cells of urinary bladder epithelium endocytosis is peculiar because it represents a mechanism only for removal of the luminal plasmalemma. The aim of this study was to identify different cytochemical markers within endocytic compartments of these cells. By the presence of fluid-phase endocytic tracer HRP variety of endocytic compartments was identified. They included endocytic vesicles beneath the luminal plasmalemma, pieomorphic structures in the apical cytoplasm and multivesicular bodies in the perinuclear region of cells. Membrane glycoproteins were analyzed by freeze-fracture cytochemistry using WGA-ferritin. Luminal plasmalemma as well as membranes of all endocytic compartments were labelled. Polyclonal antibodies raised against the asymmetric unit membrane labelled luminal plasmalemma, and limiting membranes of multivesicular bodies. The small GTP-binding protein rab4 that associates with early endosomes was localized at structures in the apical cytoplasm. The visualization of acidic organelles by electron microscopy with DAMP revealed that some multivesicular bodies are acidic. This study demonstrated different endocytic compartments that were functionally related to multivesicular bodies, which probably represented late endosomes.

Tu-173

ULTRASTRUCTURAL AND CYTOCHEMICAL OBSERVATIONS ON THE GOLGI COMPLEX OF MOLLUSCAN DIGESTIVE CELLS.*

Y. Robledo, I. Marigómez, E. Angulo and M. P. Cajaraville,

Biología Zelularra eta Zientzia Morfológiako Saila, UPV/EHU, 644 PK, E-48080 Bilbo.

The Golgi apparatus is a highly polarized structure showing several functionally different compartments. A number of cytochemical markers of these compartments have been identified in most vertebrate cell types. However, yet it is not known whether these markers are also useful to identify different Golgi compartments in invertebrate cell systems. The present study was performed to investigate the morphology and cytochemistry of the Golgi complex in the digestive cells of bivalve molluscs. As shown by TEM, the Golgi complex is located in the supranuclear portion of the cells and consists of a variable number of stacks showing several flattened cisternae, usually 3-4, but it is also usual to find isolated Golgi cisternae, depending on the digestive phase of the cell, the season and the nutritional status. The organelles are usually cup-shaped and there are numerous small vesicles with variable electron density located in the vicinity of the concave portion, identified as the *trans* face of the organelle. The *trans*-most portion of the Golgi complex is often dilated to form a spherical vesicle with electron dense materials inside. The very pleomorphic ends of the Golgi cisternae appear characteristically dilated and contain tubular structures extending across the rims. The same structures were also found in isolated vesicles located close to the Golgi bodies and presumably derived from them. The possible relationship of the tubule-containing dilations with the biogenesis of lysosomes is discussed. In addition, we performed a cytochemical study of several Golgi related enzymes: AcPase, NADPase and TPPase, as well as osmium impregnation techniques. The results indicate that AcPase activity is restricted to the *trans*-most portion of the Golgi complex and related vesicles. By contrast, TPPase and osmium impregnation show no selective compartmentalization. Furthermore, no NADPase activity could be demonstrated in Golgi bodies or in other associated structures.

*Work funded by project n° UPV 075.327-EA033/92.

Tu-175

IN VITRO FUSION OF EARLY AND LATE RAT LIVER ENDOSOMES

J. Schmid^a, R. Murphy^b, S. Schmid^c and R. Fuchs^a.

^aDept. Gen. Exp. Pathology, Univ. Vienna, Austria,

^bDept. Biol. Sci., Carnegie Mellon Univ., Pittsburgh,

and ^cDept. Mol. Biol., Scripps Research Inst., La Jolla, USA.

Regulation of endocytic membrane traffic has been studied by reconstituting fusion events between endocytic vesicles *in vitro*. Little is known, however, about the biochemical requirements for fusion of early and late endosomes derived from polarized cells of a tissue such as the liver. Therefore, we investigated the mechanism of rat liver endosome fusion in a cell-free system. Endosome labeling was carried out by perfusion of isolated rat livers with endocytic markers. Early or late endosomes of one liver were selectively labeled with biotinylated asialoorosomucoid (*b*-ASOR), a ligand for the hepatic asialoglycoprotein receptor. In a second liver, a fluid phase marker, avidin, was endocytosed into early endosomes. Incubation of endocytic vesicles from the two livers resulted in endosome fusion as indicated by formation of avidin-*b*-ASOR complexes. In agreement with endosome fusion from tissue culture cells, rat liver endosome fusion was temperature, ATP and cytosol dependent. However, fusion occurred with the same efficiency and similar kinetics between early/early as well as early/late endosomes in the absence of intact microtubules. In addition, fusion seemed to be independent of monovalent cations and anions present in the fusion buffer. Thus, rat liver endosome fusion exhibits striking differences to endocytic fusion events in tissue culture cells. Preliminary experiments revealed that single-organelle flow analysis (SOFA) can be applied to monitor endosome fusion events. FITC-dextran labeled rat liver endosomes were incubated with cytosol and ATP for different periods of time and subsequently analyzed by SOFA. During incubation the occurrence of a population of vesicles with higher side scatter was observed, indicating an increase in vesicle size. These data show that endosomal fusion events generate larger vesicles, that can be identified by SOFA. Supported by FWF P 8435.

Tu-174

COMPARTMENTALIZATION AND DIFFERENTIATION OF THE GOLGI APPARATUS IN MUSCLE FIBERS DURING DEVELOPMENT OF THE MOUSE DIAPHRAGM

C. Antony¹, M. Huchet², J.P. Changeux² et J. Cartaud¹. Institut Jacques Monod, Paris¹, Institut Pasteur, Paris², France.

Shortly after innervation of embryonic (E) diaphragms (E 13-14), a compartmentalized expression of the genes encoding acetylcholine receptor subunits appears (Piette et al, Dev. Biol. 157: 205, 1993). In myoblasts and myotubes, the Golgi apparatus (GA) is found associated with every nuclei with a typical perinuclear organization in myotubes. In adult rat, on the contrary, the GA is restricted within the subsynaptic domain of the endplate as previously shown in chicken (Jasmin et al., PNAS, 86: 7218, 1989) and rat (Jasmin et al., 1994, submitted). In the present work, we analyzed the distribution of the GA versus the synapses location in the developing diaphragms at various times after innervation using a panel of Golgi markers covering the different subcompartments of the organelle. We show that i) compartmentalization of the GA occurs at E 16, i.e. 2-3 days after innervation, ii) some Golgi markers (TGN38, Mannosidase II), although detected in fusing myoblasts at the edge of the fibers, are no longer detected in innervated fibers even beneath the subsynaptic membrane. Therefore, it appears that in innervated muscle fibers the organization of the secretory pathway is placed under the nerve control. Also a biochemical differentiation of the subsynaptic-associated GA is observed, thus suggesting that in muscle cells the GA becomes specialized upon innervation.

Tu-176

VIRUS-MEDIATED RELEASE OF ENDOSOMAL CONTENT *IN VITRO*: DIFFERENT BEHAVIOUR OF ADENOVIRUS AND RHINOVIRUS 2.

E. Prehla^a, +C. Plank^b, +E. Wagner^b, *D. Blaas^c, and R. Fuchs^a.

^aDept. Gen. Exp. Pathol., Univ. Vienna, ^bInst. Mol. Pathol., and ^cInst. Biochem., Univ. Vienna, Vienna, Austria.

Mechanisms by which non-enveloped viruses penetrate cell membranes as an early step in infection are not well understood. Current ideas about the mode for cytosolic penetration by nonenveloped viruses from endosomes include 1) local breakdown of the endosomal membrane providing direct access of infecting virions to the cytoplasm or 2) formation of a membrane-spanning pore by capsid proteins through which viral components can enter the cytoplasm, both being triggered by low pH-mediated conformational changes of capsid proteins. As a general approach to investigate the interaction of viral proteins or peptides with the endosomal membrane we have developed an *in vitro* assay, that monitors the release of a biotinylated cointernal marker (B-dextran) of varying molecular weight from isolated endosomes. Therefore, virus (Adenovirus DL 312 or Rhinovirus 2 (HRV2)) or N-terminal Influenza haemagglutinin fusogenic peptides (HA-2-peptide) was co-interned with B-dextran in the presence of Bafilomycin A1, a specific inhibitor of the endosomal proton pump, to inhibit the conformational change. Endosomes were isolated and the conformational change of viral proteins was induced by endosome acidification *in vitro*. Subsequent release of B-dextran from endosomes was quantitated by ELISA. Using this assay, we could demonstrate low pH dependent release of 10 kD B-dextran for Adenovirus (80%), HRV2 (18%), and HA-2-peptides (45%). HA-2-peptides and Adenovirus did not show a significant size-selectivity, proposing a membrane-disrupting activity, which is consistent with earlier results by FitzGerald et al. (1983, Cell 32: 607-617). In the presence of HRV 2, however, 18 % of the 10 kD dextran and undetectable amounts of the 70 kD dextran were released. Thus, the release of HRV 2-RNA into the cytosol might occur via a specific pore-forming mechanism, in contrast to the membrane-disrupting mechanism of Adenovirus. Supported by Jubiläumsfonds der Österreichischen Nationalbank Nr. 4498.

PRIMARY STRUCTURE OF MG-160, A SIALOGLYCOPROTEIN OF THE GOLGI APPARATUS.
Tu-177 J. Gonatas, Z. Mourelatos, A. Stieber and N. Gonatas.

Department of Pathology and Laboratory Medicine,
 University of Pennsylvania School of Medicine, Philadelphia, PA,
 19104, USA.

MG-160 is a 160 kDa membrane sialoglycoprotein residing in the middle cisternae of the Golgi apparatus of several rat and pheochromocytoma (PC-12) cells (Gonatas J O, et. al. *J. Biol. Chem.* 264:646-653, 1989). The primary structure of MG-160, deduced from cDNA cloning and sequencing, consists of a polypeptide of 1,171 amino acids with an M_r of 133,403 Daltons. The protein contains an intraluminal cleavable signal peptide, followed by a Pro-Gln rich segment and sixteen contiguous, ca.60 residue-long, regularly spaced cysteine-rich segments showing sequence identities ranging from 15 to 35 percent. The luminal domain is followed by a single membrane spanning domain and a short carboxy-terminal cytoplasmic tail. The protein contains 5 potential NXT glycosylation sites. The primary structure of MG-160 is consistent with previous studies which revealed that the protein displays intrachain disulfide bond, Asn-linked carbohydrates and a short cytoplasmic domain.

The primary structure of MG-160 is 90% identical to a receptor for acidic and basic fibroblast growth factors (bFGF), isolated from chicken embryos (Burris, et. al. *Mol. Biol. Cell* 12:5600-5609, 1992). MG-160 binds recombinant human basic fibroblast growth factor(bFGF), but is expressed only in the Golgi apparatus of chicken embryos. Furthermore, bFGF binds only to surfaces of chicken embryo cells and is internalized exclusively into endosomes-lysosomes of PC-12 cells. MG-160 shows no homology with members of the family of fibroblast growth factor receptors involved in signal transduction. The implications of these findings are intriguing. It remains to be elucidated whether binding to bFGF plays a principal role in the function of MG-160.

Tu-179
Control of the N-linked glycoprotein traffic at the exit of the endoplasmic reticulum in intestinal epithelial cells.

Ogier-Denis E.¹, Houri J.J.¹, De Stefanis D.², Bauvy C.¹, Baccino F.M.², Isidoro C.² & Codogno P.¹
¹. INSERM U410 Paris France. 2- University of Torino, Italy.

We have shown previously that the processing of asparagine-linked oligosaccharides is dependent on the state of enterocytic differentiation of HT-29 cells (a human colon adenocarcinoma cell line). The pivotal observation was an impairment of the trimming of high mannose glycans in undifferentiated HT-29 cells (a human colon adenocarcinoma cell line) (*J.Biol.Chem.* 263, 6031, 1988; *J.Biol.Chem.* 265, 5366, 1990). This dramatic decrease in N-glycan processing is dependent on the bypass of the Golgi apparatus by newly synthesized glycoproteins and their subsequent deposit in an acidic degradative compartment. This abnormal glycoprotein traffic was not observed in differentiated HT-29 cells (*J.Biol.Chem.* 266, 20849, 1991). More recently, we have gained further insights into the lysosomal nature of the degradative compartment and the autophagic-lysosomal pathway that leads to the missorting of glycoproteins in undifferentiated HT-29 cells (*Eur.J.Biochem.* 205, 1169, 1992; *Biochem.Biophys.Res.Commun.* 197, 805, 1993). In the present study, we show that : 1-the autophagic sequestration of endoplasmic reticulum markers is concomitant to that of high mannose substituted glycoproteins. 2-the fate of high mannose glycoproteins at the exit of the endoplasmic reticulum in undifferentiated cells (movement to the Golgi apparatus vs autophagic sequestration) is under the control of a heterotrimeric G protein Gi.

ENDOCYTOSIS RICIN IS TARGETED TO THE ENDOPLASMIC RETICULUM IN NONDIFFERENTIATED HT-29 CELLS BUT TO THE GOLGI BODY IN DIFFERENTIATED HT-29 CELLS
Tu-178

Bénédicte CHAZAUD, Marie-Paule MURIEL, Michèle AUBERY, and Monique DECASTEL

INSERM U 180, CNRS UAC 71, Centre Universitaire des Saints-Pères, 45 Rue des Saints-Pères, 75006 PARIS, FRANCE.

The aim of our study was to characterize the pathway and the translocation site for ricin, a toxic protein, in undifferentiated and differentiated HT-29 cells. Using subcellular fractionation and electron microscopy, we showed that, regardless the incubation time, internalized either radio-labelled ricin or horseradish-peroxidase conjugated ricin was targeted to the Golgi apparatus before intoxication of differentiated HT-29 cells. In contrast, in undifferentiated HT-29 cells, following short time incubation, these two compounds were found in the endoplasmic reticulum (ER), whereas for long time internalization, ricin is detected mainly in the Golgi. However, the ER is the site from which ricin exerts its most toxic activity. Upon BFA-treatment, undifferentiated HT-29 cells did not exhibit the stereotyped tubulo-vesicular Golgi-reticulum structure; the Golgi was completely vesiculated, and fused with the ER only in a minor extent. Our biochemical and morphological data indicate that, unlike in differentiated HT-29 cells, both the salvage compartment and the Golgi apparatus exhibit abnormality in their morphology, dynamics, functioning and relationship to cytoskeletal elements.

Effect Of Sulfur Mustard On Electrophoretic Mobility (EPM) Of Erythrocyte
Tu-180 N.Maghsoodi & H.Tavakoli
 I.H.University, P.O.Box 16765-1717, Tehran
 Iran

Normal cells are negatively charged at their surface and in a medium and presence of electric field migrate towards positive electrode. This surface charge is essential for cellular biophysical and physiological properties. In this study effect of mustard(which can actively bind and alkylate negatively charged molecules) on EPM of human erythrocyte was assessed.

For this purpose a microelectrophoresis device was designed and standardized(1). Human derived erythrocytes were added to its saline filled vertical cylinder and $EPM = K.(T_1-T_2) / (T_1.T_2)$ was measured without and with mustard (at different concentrations) and at different pH's (2.4, 7.4 and 12.4).

Observations revealed that EPM of the cells changed with different concentration of mustard and at different pH's. By increasing the mustard concentration in an acidic and neutral pH, EPM decreased whereas in basic pH by increasing the concentration of mustard, EPM also increased. Significance of the results will be discussed.

I-J.Bauer, Electrophoretic separation of cells. *J.Chromatography*, 418(1987) 359-383.

Tu-181 LACTOFERRIN-DEPENDENT IRON UPTAKE IN
TRITRICHOMONAS FOETUS

J. Tachezy^a, J. Kulda^a and J. Schrevel^b

^aDepartment of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic; ^bLaboratory of Parasite Biology, Protistology, Helmintology, National Museum of Natural History, Paris, France

Acquisition of lactoferrin (Lf) and iron uptake by a parasitic protozoon *Tritrichomonas foetus* has been studied. Iodinated lactoferrin (¹²⁵I-Lf) bound to the outer membrane of *T. foetus* at 4°C in a specific, time dependent and saturable fashion. About 1.7×10^5 binding sites of a single class with $K_d \approx 3.6 \mu\text{M}$ was estimated by means of Scatchard analysis. Binding of ¹²⁵I-Lf was not inhibited by an excess of non-labeled Lf glycopeptides suggesting that a polypeptide chain of the molecule was involved in Lf-binding site interaction. Internalization of the bound Lf was observed at 37°C. The cell-associated radioactivity was about 3.5-fold higher after 30 min. incubation of the parasite with ¹²⁵I-Lf at 37°C than the amount bound at 4°C. The ability of the cells to remove and accumulate iron from Lf was examined using ⁵⁹Fe-saturated Lf. The cell acquired 492 pmol ⁵⁹Fe per mg during 60 min. incubation in the presence of 100 µg/ml Lf at 37°C. Treatment of trichomonads by monensin, an agent that raise the pH of an acidic compartment, decreased iron accumulation from Lf to 80%.

In conclusion, these experiments indicate that *T. foetus* possesses specific mechanism for Lf-dependent iron uptake. The binding and internalization of Lf suggest that receptor-mediated endocytosis of Lf might be involved in this process.

TYPGENESIS OF MEMBRANE MICROHETEROGENEITY
BY CYTOSKELETON-INTERFACE LINKAGE

Tu-183 G. Isenberg

Biophysics Dept. E-22: Technical University of Munich, D-85747 Garching, FRG

Many of the known actin binding proteins have been recognized to interact with lipids (Isenberg and Goldmann, in: "The Cytoskeleton", Jai Press N.Y. 1995). Lipid binding could be important for (1) mediating anchorage of the actin skeleton to lipid bilayers; (2) for defining a specific membrane topology and (3) for regulating the function of actin binding proteins in a limited region.

I will depict two actin binding proteins: the homodimeric actin nucleating protein talin, known from focal contacts and leading edge cell membranes as well as the actin crosslinking protein filamin, to demonstrate how these proteins can be reconstituted into lipid mono- and bilayers as functional lipid-protein complexes.

PARTICIPATION OF Ca^{2+} -BINDING 40-kDa GLYCOPROTEIN IN ELECTROGENIC TRANSPORT OF CALCIUM BY MITOCHONDRIA

Tu-182

L.P. Dolgacheva

Institute of Cell Biophysics RAS,
Pushchino, Moscow Region, Russia

Mitochondrial glycoproteins (including Ca^{2+} -binding 40 kDa glycoprotein) have been isolated from rat liver mitochondria by hypotonic sucrose solution.

This treatment decreased Ca^{2+} -transport up to 50% but did not change oxidative phosphorylation rate.

The content of 40 kDa glycoprotein was measured in the intact and depleted mitochondria as well as in the hypotonic extract by immunochemical methods and was equal to 0.8%; 0.43%; 6% of total protein, respectively.

The Ca^{2+} -binding 40 kDa glycoprotein from beef heart mitochondria was used for reconstruction of Ca^{2+} -transport, since this glycoprotein is not species- or tissue-specific.

Addition of purified 40 kDa glycoprotein to the depleted mitochondria resulted in restoration of Ca^{2+} -transport up to 90% and did not exert any influence on oxidative phosphorylation rate.

This results indicate that the Ca^{2+} -binding 40 kDa glycoprotein is involved in electrogenic Ca^{2+} -transport by mitochondria.

COILIN, GOLGINS AND SPLICING FACTORS: HUMAN ANTIBODY IN EXPRESSION CLONING

Tu-184

Edward K.L. Chan, Saeko Takano, Marvin J. Fritzler¹, Haruhiko Imai, Kiyoshi Furuta and Eng M. Tan

W.M. Keck Autoimmune Disease Center, The Scripps Research Institute, La Jolla, CA 92037 USA; ¹The McCaig Center for Joint Injury and Arthritis Research, University of Calgary, Calgary, Canada T2N 4M1

Over the past few years, our laboratory has taken advantage of human autoantibodies as probes for the identification and cloning of cDNAs encoding proteins from various subcellular structures.

1. Coiled bodies (CBs) are non-capsular nuclear bodies with a diameter of 0.3-1 µm and may be sites for processing and storage of snRNPs/snoRNPs. Human anti-CB sera recognize an 80-kD phosphoprotein named p80-coilin. With human autoantibody as probe, p80-coilin cDNAs were cloned and shown to have sequence similar to that of SPH-1, an 80-kD protein of the spheres in *Xenopus* oocytes.

2. Autoantibodies to the Golgi complex have been used to obtain 2 unique cDNAs encoding 95-kD and 160-kD proteins that share significant sequence similarity. These proteins, golgin-95 and golgin-165, may be members of a coiled-coil protein family in the Golgi complex (Fritzler *et al.*, *J. Exp. Med.* 178:49-62, 1993).

3. The HCC1 protein was identified using autoantibodies from a patient who had pre-existing liver disease that eventually developed into hepatocellular carcinoma (HCC) (Imai *et al.*, *J. Clin. Invest.* 92:2419-2426, 1993). HCC1 (64kD) was shown to contain two classes of motifs found in several splicing factors: an N-terminal SR domain, which has 6 contiguous octapeptide repeats (RS-ERK motif), and 3 RNA-binding protein domains in the C-terminal two-third of HCC1. In immunofluorescence microscopy, affinity purified antibodies to HCC1 give a nuclear speckled-network that co-localizes with non-snRNP splicing factor SC35.

AUTOANTIBODIES AS PROBES: THE VIEWPOINT OF AN IMMUNOLOGIST

Tu-185 K.M. Pollard¹

¹W.M. Keck Autoimmune Disease Center, Dept. of Molecular & Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA.

Molecular and cell biologists have found autoantibodies useful reporter molecules for probing structure/function relationships within the cell, particularly the cell nucleus. For the immunologist, autoantibodies also serve as markers but the perspective differs from that of the biologist. For the immunologist, most autoantibodies are viewed as the endpoint of autoimmunity. The central unanswered question being the mechanism leading to auto-antibody production. Autoantibodies react with non-denatured, conserved, and often functional regions of autoantigens: features not common to antibodies elicited by deliberate immunization. In the systemic autoimmune diseases autoantibodies are not thought the causative agent of disease, but can serve as diagnostic markers. Some autoantibody/disease relationships, such as the anti-nucleolar autoantibodies of scleroderma, point provocatively to a cellular organelle as the stimulus of the autoantibody response. Chemicals can perturb the immune system sufficiently to elicit autoantibodies of restricted specificity in both man and animals, such as the anti-nucleolar autoantibodies produced in mice given heavy metals. Animal models of chemical-induced autoimmunity, in which the eliciting chemical and the auto-antibody target are known, offer great potential for probing the pathway(s) leading to autoantibody production.

AN ALTERED EXPRESSION OF THE

Tu-186 GENE ENCODING FOR THE NUCLEAR AUTOANTIGEN LA/SS-B

H. Tröster and M. Bachmann, Inst. Phys. Chem., Jo.-Gu.-Uni. Mainz, F.R.G.

Sera of patients with SLE and pSS frequently contain autoantibodies to the nuclear autoantigen La/SS-B. Two different forms of La mRNAs can be expressed from the same La gene using two different promotor sites. One of the promotores locates upstream of the exon1 while the other locates within the intron downstream of the exon1. If the promotor within the intron is used then a kryptic splice site within the intron is used for an alternative splicing process resulting in the exchange of the complete 5'-terminal exon1 sequence. The alternative 5'-terminus contains three further AUGs. Using the technique of confocal Laser Scanning Microscopy combined with digital image processing, we analyzed the expression of La protein by *in situ* hybridizations and immunolocalizations comparing the staining patterns of patient's anti-La antibodies immunoadsorbed to recombinant human La protein and monoclonal anti-La and anti-Ro52 antibodies.

NOR-PROTEINS DURING MITOSIS

Tu-187 P. Roussel and D. Hernandez-Verdun
Institut Jacques Monod, 2 place Jussieu, 75251
Paris Cedex 05 France

During mitosis, the ribosomal genes are clustered in the secondary constriction of chromosomes (NORs) in association with non-histone proteins, the NOR-proteins. To understand the steps necessary for the assembly and disassembly of nucleoli during the cell cycle, the characterisation of the mitotic NOR-proteins has been undertaken. Several human autoimmune sera were used that recognized mitotic NOR-proteins. The antigens were identified on western blots and the antibodies were used to immunoprecipitate the complexes in mitotic extracts. The sera recognize RNA polymerase I transcription factor UBF, several subunits of RNA polymerase I, and factors that could participate to the SL1 complex. Immunoprecipitation showed that RNA polymerase I complexes remained assembled during mitosis. Therefore, the basic components of the transcription machinery of active ribosomal genes appear present in the NORs during mitosis.

THE NATURE OF COILED BODIES

Tu-188 I. Raška, M. Dundr, K. Koberna

Laboratory of Cell Biology, Academy of Sci. of Czech Republic, Albertov 4, Prague, Czech Republic

Coiled body (CB) is an ubiquitous nuclear RNP structure present in both plant and animal cells. It is usually of spherical appearance and is composed of coiled filaments. Although it was described almost 100 years ago by Ramon y Cajal, its function is yet unknown. In order to deepen the knowledge about this nuclear organelle, we have performed the light and EM affinity cytochemistry study on CBs. We have implemented a number of affinity cytochemistry probes primarily on cultured mammalian cells, the reference probe being the antibody to coilin, the specific 80-kD protein of CBs. Whereas no detectable quantities of DNA were demonstrated within CBs, the most striking feature about the CBs is probably their enrichment in snRNAs and snoRNAs, respectively in snRNPs and snoRNPs. According to their behaviour under various experimental conditions (an alteration of the cell growth, alterations of DNA, RNA or protein metabolism), we consider CBs as markers of the cell metabolism. Their presence in the cell reflects, however, rather metabolic disbalance within the cell nucleus. The conclusion that CBs do not represent specialized splicing domains is also supported by the absence of label due to a nonisotopic detection of incorporated bromouridine. We are of the opinion that CBs are primarily involved in the metabolism of snRNPs and snoRNPs, likely in their maturation, storage and recycling. Such functions do not exclude a possible transport role played by CBs. In addition, and in harmony with the above proposal, we consider CBs as reperfusion site of active processes, such as locally ongoing intense RNA synthesis, situated nearby.

NATURAL ANTIBODIES: PROPERTIES, DISTRIBUTION,
INTERACTION WITH CELL STRUCTURES.

Tu-189 A.M.Poverennyy, V.A.Saenko and A.E.Kabakov

Department of Biochemistry, Medical Radiological Research
Center RAMS, Oboinsk, Russia

One of the properties of certain natural antibodies is polyspecificity towards the ligands displayed as ability to interact with DNA and other polyanions.

Natural high avidity antibodies reacting with native DNA and those to cardiolipin were demonstrated to be masked in healthy individuals sera. Their activity can be revealed after ion exchange chromatography on anion-exchanger or other special procedures. Binding of some populations of antibodies to DNA was found to be cofactor-dependent. Antibodies reacting with DNA and different polyanions interact with certain cellular proteins of different molecular weight. Immunomorphological studies demonstrated that antibodies interacting with proteins of different molecular weight can react with different cellular structures.

SPECTRIN IN FUNGI AND PROTOZOA

Tu-190

O. Nečas, R. Janisch, I. Pokorná,
M. Gabriel, M. Kopecká and A. Svoboda

Department of Biology, Faculty of Medicine,
Masaryk University, 662 43 Brno, Czech Republic

Spectrin, the major protein of the membrane cytoskeleton, is common to all animal cells. It has already been identified in some plant cells and amoebas but there is no evidence of its existence in fungi and infusoria. Its proof by indirect immunofluorescence in selected fungal species (*Saccharomyces cerevisiae*, *Schizosaccharomyces versatilis* and *Rhizopus nigricans*) and the infusorian *Paramecium caudatum* was the objective of this study. Anti-chicken erythrocyte spectrin antibodies demonstrated spectrin both in submembraneous regions and intracellularly in yeast cells, protoplasts and yeast zygotes, while in *Rhizopus* spores spectrin was localized only submembraneously. Growing regions, i.e., small buds, conjugation projections, growing protoplasts and hyphae, showed only a very weak or no fluorescence. In *Paramecium caudatum*, spectrin was detected in several membrane structures of the cortex, such as ridges enclosing the kinetids, i.e., the sites where the granulofibrillar meshwork was located immediately under the plasma membrane, then in the alveolar epiplasm, ciliary plasma membrane and in the membranes of contractile vacuole pores and cytoproct.

COLD SENSITIVITY OF MICROTUBULES IN
Tu-191 YEASTS

O. Nečas, M. Krejčí-Pavliková, V. Urbánek, K. Kaňková and J. Kalabis

Department of Biology, Faculty of Medicine,
Masaryk University, 662 43 Brno, Czech Republic

There is no comprehensive study on the sensitivity of microtubules (MT) to low temperature in fungi, a class of organisms living within a broad range of physiological temperatures. The cycling cells of *Saccharomyces cerevisiae* (*S. cer.*) and *Schizosaccharomyces versatilis* (*Sch. ver.*) were subjected to a cold shock by a sudden drop of temperature down to 0°C (10°C per sec.). MT were visualized by indirect immunofluorescence using TU 01 (against alpha tubulin) and TAT (against alpha tubulin) antibodies in *S. cer.* and *Sch. ver.*, respectively. In *S. cer.*, cytoplasmic MTs depolymerized while spindle MTs were resistant. Spindle pole bodies (SPB) also showed fluorescence. The critical temperature was at 3°C. A complete repolymerization of cytoplasmic MTs emanating from the SPB occurred as a result of a rapid increase in temperature up to 20°C in 60 sec. In *Sch. ver.* both cytoplasmic and spindle MTs were sensitive to cold. Their repolymerization proceeded gradually during a 5 min period through randomly localized short MTs. When temperature was decreased at a lower rate (1°C per sec.), the cytoplasmic MTs could be detected only in some cells of the population studied.

THE EFFECT OF ISOPROPYL N-(3-CHLOROPHENYL)-CARBAMATE ON YEAST CELLS OF
Tu-192 YARROVIA LIPOLYTICA

M. Havelková^a, I. Hönes^b, K. Augsten^b,
E. Unger^b and A. Svoboda^a

^aDepartment of Biology, Faculty of Medicine,
Masaryk University, 662 43 Brno, Czech Republic;

^bDepartment of Molecular Biotechnology, Jena,
Germany

Isopropyl N-(3-chlorophenyl)-carbamate (CIPS) is a microtubular toxin. The effect of CIPS on the growth and morphology of *Y. lipolytica* cells, especially with respect to nuclear and cytoskeletal components, was investigated during 6 h of incubation. CIPS (50 µm/ml) added to nutrient medium arrested the cell cycle. After the 1st h of incubation with CIPS, the majority of cultured cells showed a single nucleus. Microtubules were reduced to spindle pole bodies (SPB) in most of the cells. These duplicated but not separated. In about 15% of the population, short thin cytoplasmic microtubules extended from the SPBs. Nuclear microtubules were absent. Actin structures involved only actin patches; actin cables were missing. The cytoplasm contained numerous small vesicles and short membranes. At 2 h of incubation with CIPS, actin patches decreased in number and became delocalized. This implies that the CIPS effect was primarily aimed at microtubules. Only after the microtubules were disassembled, reduction and delocalization of actin structures occurred. As a result, exocytosis was blocked and exocytotic vesicles accumulated in the cytoplasm.

Tu-193 ASSOCIATION OF LIPID DROPLETS AND MITOCHONDRIA TO CYTOSKELETAL STRUCTURES IN CULTURED MOUSE LEYDIG CELLS
B. Bilinska
Laboratory of Animal Endocrinology and Tissue Culture, Institute of Zoology, Jagiellonian University, Krakow, Poland

The initial steps of steroidogenesis involve the transport of cholesterol from lipid droplets to the inner mitochondrial membrane where steroidogenesis begins by conversion of cholesterol to pregnenolone. Cytoskeleton is suggested to be involved in steroidogenesis, thus the localization of lipid droplets and mitochondria in relation to cytoskeletal networks seems to be of special interest. Leydig cells obtained from testes of mature Swiss mice were routinely purified by a Percoll density gradient and cultured during 48 hrs. Immunofluorescence studies were carried out using monoclonal antibodies against tubulin, vimentin followed by FITC and showed typical network of microtubules and vimentin intermediate filaments. Microfilaments were detected by rhodamine-phalloidin and were organized as stress fibres. Mitochondria and lipid droplets were visualized using anilinonaphthalene-sulphate (ANS) fluorescent dye and were observed as radial and elongated structures, respectively.

Incubation in the presence of cytoskeletal disrupting drugs and I.H. resulted in the increase of testosterone secretion with concomitant changes of cell shape towards more regular and rounded. Mitochondria were brought into a closer proximity with cytoskeletal structures, suggesting functional importance of these cellular constituents in possible regulation of cholesterol transport.

Tu-195 CYTOSKELETAL DYNAMICS IN MICROINJECTED PLANT CELLS
C.W. Lloyd^a, P.J. Shaw^a, C.J. Staiger^a, R.M. Warn^b and M. Yuan^b
^aDepartment of Cell Biology, The John Innes Institute, Norwich, UK; ^bSchool of Biological Sciences, the University of East Anglia, Norwich, UK.

Microinjection of walled plant cells reveals the cytoskeleton to be much more dynamic than was suspected. Transverse microtubules are found in elongating cells but the switch to longitudinal alignment encourages lateral swelling. By labelling transverse microtubules with microinjected rhodamine-tubulin we have directly observed this 90° reorientation. The speed of fluorescence recovery after photobleaching confirms that these microtubules are not stable but display properties consistent with dynamic instability. The ability of plant microtubules to dynamically restructure their arrays accounts for rapid responses to a range of physiological factors, including gibberellic acid, abscisic acid, auxin and light.

In other experiments we have microinjected recombinant plant profilin (from R. Valenta) into *Tradescantia* stamen hair cells. Within minutes, this actin-binding protein causes cytoplasmic strands to snap and cytoplasmic streaming to stop. Second injections with rhodamine phalloidin indicated this was due to depolymerization of F-actin. This effect could be mimicked with another G-actin binding protein, DNase I, suggesting that F-actin cables in plants are in dynamic equilibrium with the G-actin pool.

Tu-194 EFFECT OF EPIDERMAL GROWTH FACTOR (EGF) ON PHAGOSOME-LYSOSOME (P-L) FUSION AND ON F-ACTIN CONTENT IN PERITONEAL MACROPHAGES OF MICE
T. Mozhenok, L. Solovjeva, A. Braun, A. Bulychev

Laboratory of Biochemical Cytology and Cytochemistry, Institute of Cytology of the RAN, St. Petersburg, Russia.

P-L fusion has a key position in the endocytosis. EGF plays an essential role in a wide variety of cellular processes such as chemotaxis, differentiation, endocytosis, etc. It is well known that EGF undergoes metabolic degradation in lysosomes. There are observations of EGF influence on cytoskeleton.

Vital dye acridine orange (AO) and fluorescent phalloidin (FITC-phalloidin) were used in this study. Observations were carried out on fluorescent microscope LUMAM-111 (LOMO). The content of F-actin was measured on inverted fluorescent microscope ICM-405 (Opton), controlled by computer MOP-VIDEOPLAN (Rontron). EGF was used at concentrations 100, 150 and 300 ng/ml.

Our results showed that EGF stimulated P-L fusion and increased F-actin content. Effect of EGF is dependent on its concentration. EGF changed localization of F-actin in cell. In control cells F-actin distributed diffusely; at the action of EGF F-actin aggregated on the cell pole. Comparison of the received data suggests a correlation between the intensity of P-L fusion and F-actin content in macrophages at the action of EGF.

Tu-196 IMMUNOELECTRON MICROSCOPICAL LOCALIZATION OF DIFFERENT PROTEINS IN THE CENTROMERE REGION.

N. Paweletz, D. Schroeter, M. Knehr, E.-M. Finze, U.-L. Kiesewetter

German Cancer Research Center, Dept. 430, 69120 Heidelberg, Im Neuenheimer Feld 280, FRG.

Sera from Scleroderma patients, recognizing the centromere/kinetochore complex of HeLa chromosomes in immunofluorescence microscopy were used to localize the antigen binding sites by electron microscopy, applying gold label and silver enhancement. Mitotic HeLa cells were selected by shaking off and reattached or spun down to polylysine coated slides. They were stained before embedding.

A commercial centromere control serum binds to a protein below the kinetochore, similar to CENP-B. Serum LA precipitates proteins in the inner layer of the kinetochore comparable to CENP-C. Serum SL detects an antigen covering and penetrating the centromere, and serum DI facultatively labels the centromere (pro- and metaphase) or the midbody (ana- and telophase). The reactive proteins are determined by western blots.

We suggest, the identification of such proteins in the kinetochore and at or in the centromere can help to disclose their function during mitotic chromosome transport and separation.

MAP2 DURING CAT BRAIN DEVELOPMENT:
Influence of phosphorylation and conformation.

Tu-197 B.M. Riederer^a, E. Dráberová^b, P. Dráber^b, and
V. Viklický^b

^aInstitute of Anatomy, 1005 Lausanne, Switzerland; ^b Institute of Genetics, Praha, Czech Republic,

Microtubule-associated protein 2 (MAP2) is an essential protein for the stability and rigidity of microtubules. In cat brain it exists as two variants, MAP2b and MAP2c (Riederer and Innocenti, Eur. J. Neurosci. 4: 1376-1386, 1992). Five monoclonal antibodies were used to study the immunocytochemical distribution during cat cortex and cerebellum development. In contrast to monoclonal C, AP18 reacted with a phosphorylated site and indicated that MAP2b and 2c are phosphorylated throughout postnatal development. The AP18 epitope was less susceptible to dephosphorylation in larger apical dendrites of cortical pyramidal cells. Antibodies AP14, MT01, MT02 detected MAP2b only. During the first postnatal week, staining of cellular elements in cerebellum differed between these antibodies, but at later ages similar neuronal elements were stained. Interestingly, MT02 stained at early stages cell bodies and dendrites in cerebral cortex and cerebellum. With progressing maturation the immunoreactivity became restricted to distal parts of apical dendrites of pyramidal cells. In contrast to other monoclonals, MT02 immunostaining decreased in developing cerebellum and was barely detectable in adult animals. Immunohistochemical detection and function of MAP2 may depend on its conformation, phosphorylation or other posttranslational modifications. This work was supported by the Swiss National Science Foundation, grants No. 31-33447.92 and 7TRPJ038608.

LIPOSOME MEDIATED GENE TRANSFER
VIA SPERM CELLS

Tu-198 O.J. Rottmann¹, R. Antes¹, P. Höfer¹,
B. Sommer² and F. Grummt²

¹ Lehrstuhl für Tierzucht, TU München-Weihenstephan, D-85350 Freising; Germany

² Institut für Biochemie, Universität Würzburg, am Hubland, D-97074 Würzburg, Germany

Spermatozoa of several species were incubated with liposomes containing plasmid DNA. Sperm cells were washed thoroughly, the genomic DNA was prepared and tested for plasmid DNA by PCR, dot blot and Southern analysis. The plasmid always was found intact within the sperm cell. When liposome treated sperm cells were artificially inseminated a high percentage of transgenic offspring resulted as proved by PCR and Southern analysis. Transgenic rabbits, tested so far, transmitted the foreign sequences to the F1. Gene expression was demonstrated quantitatively and qualitatively.

NUCLEAR TRANSLOCATION OF AN
EXOGENOUS PROTEIN CONTAINING TAT

Tu-199 REQUIRES UNFOLDING

A. Rubartelli, N. Bonifaci, L. De Benedetti and R. Sita²

Ist. Naz. Ricerca sul Cancro, Genova and "DIBIT, Milano, Italy

Tat protein from HIV-1 is a transactivation factor essential for viral replication. Unexpectedly, Tat protein can also be secreted by infected cells and taken up by target cells, where it is able to transactivate the HIV-LTR promoter in the nucleus. In order to understand whether Tat protein can drag an exogenous non nuclear protein to the nucleus of target cells, we constructed a fusion protein between tat and the cytosolic enzyme dihydrofolate reductase (DHFR). When cells transfected with the wild type Tat gene or with the fusion gene Tat-DHFR are co-cultivated with target cells harbouring the reporter gene LTR-CAT, transcellular transactivation is observed, indicating that Tat sequence behaves as a topogenic signal in readressing a differently localized protein. When HeLa cells are incubated with *in vitro* translated ³⁵S Tat or Tat-DHFR, a small amount of labeled recombinant protein becomes protected from protease digestion and is found in the nucleus of target cells. Pre-treatment of the proteins with methotrexate inhibits nuclear translocation of the chimeric protein Tat-DHFR but not of wild type Tat. As methotrexate stabilizes DHFR, this observation suggests that, as for translocation of other membranes, unfolding is required for Tat translocation to the nucleus of target cells.

HETEROGENEITY OF PEROXISOMES:
implications for peroxisome structure

Tu-200 and biogenesis

M. Wilcke,^{1,2} and S. Alexson²

¹The Wenner-Gren Institute,²Stockholm University, Stockholm, and ²Dept. of Clinical Chemistry, Huddinge University Hospital, Huddinge, Sweden

According to current concepts, new peroxisomes are formed by division of pre-existing peroxisomes or by budding from a peroxisomal reticulum. We have observed that substantial amounts of peroxisomal proteins are found together with "microsomes" (100 000 x g pellet) after subcellular fractionation of rat liver homogenates. In this study we have investigated the origin of these peroxisomal proteins by modified gradient centrifugation procedures. By comparison of sedimentation properties in Nycoadenz and Percoll gradients by analysis of enzyme activity distributions, Western blotting and immunoelectron microscopy, we concluded that most of this material is confined to novel structures of "peroxisomes". Double immuno-labelling experiments showed a strong heterogeneity in the protein contents in these vesicles and in addition identified "peroxisomes" varying in size from about 0.5 nm (normal peroxisomes") to extremely small vesicles of less than 100 nm in diameter. Our results show that we can isolate at least four classes of "peroxisomes" from livers of di(2-ethylhexyl)phthalate-treated rats that can be distinguished by different protein contents, size and sedimentation properties. These structures may possibly be related to different peroxisomal subcompartments involved in protein import.

We - 1

DEVELOPMENT OF RENAL COLLECTING DUCT: EVIDENCE FOR TRANSDIFFERENTIATION MECHANISMS
W. Röckl, S. Kloth, T. Gmeiner, J. Aigner, and W.W. Minuth
Institute of Anatomy, University of Regensburg,
93053 Regensburg, Germany

The epithelium of the collecting duct changes its function during kidney development. While the embryonic epithelium plays a key role in the process of nephron induction, the mature collecting duct takes part in controlling the acid/base status, the Na/K balance and the water content of the urine. The functions of the differentiated epithelium are maintained by distinct three cell types, which can be discriminated by morphological and physiological criteria. However, the origin of the different mature cell types as well as the nature of differentiation factors remained unclear.

By means of different newly developed monoclonal antibodies it could be demonstrated for the first time that there are considerable differences in protein expression during the maturation of the collecting duct epithelium. The mature collecting duct epithelium of the adult organism differs as well from the neonatal as from the embryonic collecting duct.

In order to investigate the transdifferentiation of the collecting duct epithelium an organotypic cell culture system was used. Embryonic collecting duct epithelium was cultured for 14 days under permanent medium perfusion (1ml/h). Monoclonal antibodies were used to monitor the expression of cell type specific proteins.

Perfusion cultured embryonic epithelium developed characteristic features of the collecting duct of in the neonatal kidney. Furthermore, application of aldosterone resulted in a further differentiation of the cultured epithelium: cells could be detected, which showed the typical protein expression pattern of fully matured collecting duct epithelium.

We - 3

VALUE OF CORRELATION OF TGF- α AND CLINICOPATHOLOGICAL PARAMETERS IN HUMAN LUNG CARCINOMA

L. Pečur^a, S. Kapitanović^a, Z. Sonicki^b, F. Pavičić^c, Š. Spaventi^d, R. Spaventi^a and K. Pavelić^a

^aDept. Molecular Medicine, R. Bošković Inst., Zagreb, Croatia; ^bA. Štampar, Medical School University of Zagreb, Croatia; ^cClinic of Pulmonary Diseases Jordanovac, Medical School University of Zagreb; ^dCroatian Academy of Sciences and Arts, Zagreb, Croatia

Despite arising data on relation of oncogenes, growth factors and their receptors in the etiology of lung cancer, in the routine diagnostic and prognostic process standard clinicopathological examinations are still used. EGF/erb-B family of peptides could play a role in lung carcinogenesis. However, despite of number studies performed, the results concerning their role in lung carcinogenesis are conflicting. In order to determine association that could be of value in clinical application we investigated relationship between TGF- α expression in 51 lung carcinomas and 26 clinicopathological parameters. None of anamnestic and pathohistological data (including grade of differentiation and tumor size) showed correlation to the level of TGF- α expression. The only significant correlation notified was the one between TGF- α and venous blood erythrocytes as well as eosinophils. Data on correlation between TGF- α and nodal status and distant metastases suggests relation of this growth factor to the aggressive biological behaviour this subset of cancer. This study shows that TGF- α expression cannot serve as an independent tumor marker for lung cancer.

EFFECT OF ALPHA-FETOPROTEIN (AFP) ON HUMAN SKIN CELLS IN VITRO

We - 2
E. Vorotelyak, A. Vasiliev, S. Gusev, and V. Terskikh

Group of Problems of Cell Proliferation,
Institute of Developmental Biology, Russian Acad. Sci.,
Vavilov Str. 26, 117808 Moscow, Russia

Now epidermal keratinocytes (EK) are considered as cells involved in immune processes. We studied the effect of immunomodulating agent AFP on proliferation of cultured EK and embryo fibroblasts and on gel contraction by these cell types. EK derived from skin fragments by cold trypsinisation were cultured in DMEM:F-12 mixture supplemented with FCS, EGF, insulin and isoproterenol. Basal cell monolayers were produced by stripping off differentiated cells after incubation of EK cultures during 2 days in Ca⁺⁺-free serum-free medium. Addition of fresh medium supplemented with Ca⁺⁺ and growth factors stimulated EK proliferation. In stimulated EK relatively small concentration of AFP (1 µg/ml) inhibited ³H-thymidine incorporation in EK in serum-free medium while 0.01 µg/ml AFP significantly inhibited thymidine incorporation in the presence of serum. AFP did not suppress proliferation of fibroblasts and even stimulated it (0.01 µg/ml). AFP inhibited gel contraction by EK while it did not affect gel contraction by fibroblasts. In living skin equivalent fibroblasts counteracted gel contraction produced by EK. We suggest that immunosuppressive agent AFP modulates behaviour of the EK in vitro.

GROWTH HORMONE RECEPTOR EXPRESSION IN HUMAN KERATINOCYTES

We - 4
D.T. Lincoln^a and E. El-Hifnawi^b

^aFaculty of Allied Health Sciences and Nursing, Kuwait University, Kuwait;
^bDepartment of Ophthalmology, Medical University of Lübeck, Germany.

Increasing evidence indicates that growth hormone (GH) can act locally and stimulates both growth and differentiation of proliferating mammalian cells, which can then synthesize insulin like growth factor I. GH is mitogenic for a variety of skin cells including fibroblasts, macrophages, adipocytes, lymphocytes and endothelial cells. A local action of GH requires the presence of GH-receptors (GH-R) which hitherto have not been demonstrated in human keratinocytes. Using well defined monoclonal antibodies reactive against the rat, rabbit and human GH-R (Lincoln et al., 1990, *Acta histochem.*, Suppl. 40, 47), this study demonstrates immunohistochemical evidence of the presence of binding sites of GH in human keratinocyte cells. A strong increase in GH-R immunoreactivity was observed between keratinocytes of normal skin and common melanocytic naevi of the junctional, epidermal, dermal and compound types. GH-R expression in keratinocytes of normal epidermis, independent from pathological tissues, was generally low or absent. On the other hand, keratinocytes in skin adjacent to or overlaying pathological tissue, had immunoreactivity of variable intensity. Furthermore, culture studies on transformed human KJD keratinocyte cells indicate that GH-R expression correlates with cell proliferation indicated by PCNA/Cyclin expression. Expression of GH-R on proliferating and on well differentiated keratinocyte cells indicates a role for GH on non-progenitor cells. GH-R immunoreactivity on differentiating and/or differentiated cells suggests that GH is also necessary for, or has a trophic function in differentiation. We propose that direct GH action is necessary not only for differentiation of progenitor cells, as implied by the dual effector hypothesis, but also for their subsequent clonal expansion, differentiation and maintenance.

We-5 ABROGATION OF PROTEINURIA IN HgCl₂-INDUCED NEPHRITIS IN THE BROWN NORWAY RAT BY TREATMENT WITH AB AGAINST TNF α

A. Molina, T. Bricio, A. Martín and F. Mampaso.
Department of Immunopathology, Hospital Ramón y Cajal, Madrid, Spain.

HgCl₂ induces an autoimmune disease in the Brown Norway (BN) rat involving synthesis of anti-glomerular basement membrane (GBM) Abs with development of nephritis and proteinuria. The administration to HgCl₂-treated rats (1 mg/kg, three times a wk) of anti-TNF α Ab (25,000 neutralizing units on days 0, 8, and 13) completely abrogates proteinuria, but circulating anti-GBM Abs and lineal glomerular IgG deposits were unmodified by the treatment. In addition, serum TNF concentration showed not significant differences after anti-TNF Ab was administered.

Group	Treatment	UProt (ng/24h)	α -GBM Ab (% binding)	Glom. depts (IF)	Serum TNF (pg/ml)
A(n=12)	HgCl ₂	134±46	160±58	+++	3.09±0.92
B(n=12)	HgCl ₂ + α -TNF	7±1*	129±45	+++	3.89±1.05
C(n=6)	H ₂ O	10±2	10±1	-	4.74±0.39

Means±SD (between days 13-15); *p<0.001.

Our results suggest the anti-TNF α administration blocks locally TNF production by intrinsic glomerular cells, thus avoiding glomerular basement membrane damage and further development of proteinuria.

We-7 SELECTIVE INTERACTIONS OF BREAST CANCER CELLS WITH STROMAL FIBROBLASTS IN CO-CULTURE

L. Adam, M. Crepin and E. Spanakis
Institut d'Oncologie Cellulaire et Moléculaire Humaine,
129 rue de Stalingrad, F-93000, BOBIGNY, France.

Using a co-culture system in which mammary epithelial cells (MCF7) and fibroblasts may communicate through a microporous membrane we demonstrate the occurrence of two types of interactions. Firstly, non-specific interactions which are to be observed with fibroblasts of any origin; the intensity of these interactions depends on the densities and the growth rates of the co-existing cell populations. Secondly, specific interactions that are dependent on the origin of the fibroblasts. Tumoral epithelial cells stimulated fibroblast growth differentially. Fibroblasts from intermediate grade tumours were stimulated more than normal fibroblasts but less than fibroblasts from advanced grade tumours. *In vivo*, this may be how transformed cells select their stromal partners among fibroblast subtypes. But, selection may also be exercised in the other direction. The fibroblasts specifically modulated epithelial phenotypic characters of prognostic value and, probably, the aggressiveness of the epithelial population. Fibroblasts from normal tissues increased the levels of progesterone-receptor protein, a good prognostic marker for breast cancer cells, presumably rendering MCF7 more responsive to steroid hormones. In contrast, fibroblasts from advanced malignant tumours increased the levels of a poor prognosis marker, the cathepsin-D protein, and, probably, the total aggressiveness of the epithelial cells.

We propose a feedback mechanism whereby growing epithelial cells cause differential growth of fibroblast variants and the selected fibroblasts cause, in turn, differential growth or death of epithelial variants. Under normal circumstances, such a mechanism accounts for a rapid co-adaptation of the tissues to the ever changing physiological environment and functional tasks of the gland. Selection for a 'wrong' variant from either tissue under particular (micro-)environmental constraints may, however, initiate a chain of co-adaptation cycles with no end, leading to tumour development.

We-6 IGF-I AND ESTROGEN RECEPTOR STATUS IN SHEEP OVARIAN GRANULOSA CELLS

M. Tománek, D. Monniaux, C. Pisselet

Animal Production Research Institute Prague-Uhříněves, Czech Republic (M.T.) and INRA, PRMD, Nouzilly, France (D.M., C.P.)

Insulin-like growth factor-I (IGF-I) exerts mitogenic and cyto-differentiative effects in ovarian follicle development and accumulated data suggest that cross-talk between peptide growth factors and steroid hormone receptors is important in regulating cell proliferation and differentiation.

Using thymidine labeling and PCNA/cyclin staining we have found that IGF-I promotes DNA synthesis and ovine granulosa cell proliferation *in vitro*. Coincidentally, up-regulation of estrogen receptor in growing cultures was determined by immunocytochemistry and Western immuno blots using monoclonal anti-ER antibodies. Blockage of IGF-I effect by tyrosine kinase inhibitor genistein caused inhibition of proliferation and decrease of ER content as determined by Western immuno blot. Paradoxically, ER immunocytochemistry revealed nuclear ER labeling in genistein inhibited cells. Cell-cycle and cytoplasmic nuclear traffic linked aspects of estrogen receptor accumulation in granulosa cells are discussed.

We-8 DIFFERENTIATION OF HUMAN NEUROBLASTOMA CELLS IN SERUM-FREE MEDIUM

J. Kremerskothen and A. Barnekow
Dept. Exp. Tumorbiology, University Muenster,
Badestraße 9, D-48149 Muenster, Germany

Neuroblastoma is a neural crest tumour and one of the most common solid tumours of early childhood. One characteristic of these tumours is their high frequency of spontaneous regression. The initial reason for this regression is so far unknown. To study the process of differentiation of neuroblastoma, the establishment of cell lines for *in vitro* studies was a very helpful tool. The adrenergic human neuroblastoma cell line SH-SY5Y is able to further differentiate along a neuronal pathway in the presence of several inducers including nanomolar concentrations of the phorbol diester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), Nerve Growth Factor (NGF) and sodium butyrate. Mouse neuroblastoma cell lines like C1300 are also known to differentiate into neuron-like cells under serum-free conditions. In our investigation we demonstrate, that prolonged culturing or incubation in serum-free medium induces drastic morphological and biochemical alterations in SH-SY5Y cells and leads to a specific differentiation into neuron-like cells.

We - 9

LOSS OF EPITHELIAL BARRIER FUNCTION IN TRANSFORMING GROWTH FACTOR-BETA-STIMULATED THYROCYTES IS PARALLELLED BY TRANSLOCATION OF CYTOPLASMIC VINCULIN TO THE ADHERENS JUNCTION

Mikael Nilsson, Lars E Ericson. Dept. Anatomy & Cell Biology, Univ. of Göteborg, Medicinaregatan 3, S-41390 Göteborg/Sweden.

The integrity of epithelia depends on the establishment and maintenance of firm cell-cell adhesion. The interaction of membrane-spanning cell-cell adhesion molecules with the cytoskeleton provides a basis of the regulation of epithelial junctions by external signals. An important component of the submembraneous cytoskeleton is vinculin which, as a target protein of e.g. phorbol ester-stimulated protein kinase C and Rous sarcoma virus kinase p60^{src}, may be involved in cell transformation. Here we have examined the effect of peptide growth factors on epithelial tightness - estimated by measuring the transepithelial resistance (R) and flux of [³H]inulin - and immunolocalization of vinculin in preformed, growth-arrested monolayers of porcine thyrocytes in Transwell culture.

Vinculin immunoreactivity in untreated cultures, which had a very high R (>6000 Ω·cm²), was distributed mainly in the cytoplasm and stained the cell borders only weakly. EGF (10 ng/ml) and IGF-1 (100 ng/ml) caused cell proliferation, thus overcoming contact inhibition, but did not change the barrier function or vinculin distribution. In contrast, TGF-β1 (10 ng/ml), which had no mitogenic effect, reduced R and increased the paracellular [³H]inulin flux after 2-3 d of stimulation. Simultaneously, an intense immunolabeling of vinculin was noted all around the cell borders at the level of the adherens junction-associated actin ring which remained. EGF but not IGF-1 promoted the TGF-β1-induced loss of the barrier function which appeared along with cell elongation and emigration from the monolayer proper. The data suggest a role of vinculin translocation to the area of cell-cell contact in TGF-β1-induced junctional dissociation preceding a change of the polarized thyroid epithelial cells to a fibroblastoid morphotype. Mitogens (EGF and IGF-1) interact differently with TGF-β1 in this response.

We - 10

66-KILODALTON PROTEIN ASSOCIATED TO EPIDERMAL GROWTH FACTOR RECEPTOR IS A PROTEOLYTIC FRAGMENT OF PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C.

V.U.Alexeyev, N.D.Medvedeva, N.V.Tsupkina and N.N.Nikolsky

Laboratory of Cell Cycle Physiology, Institute of Cytology,
Tichoretsky ave., 4, St.Petersburg, 194064, Russia

The cell signal transduction triggered by growth factors includes binding of growth factor receptors to several signaling molecules. Phospholipase Cy1 (PLCγ1) is a member of a family of PLCs that plays a certain role in cell signal transduction. PLCγ1 takes part in phosphoinositide metabolism generating simultaneously two second messengers: diacylglycerol and phosphoinositidetris-phosphate. PLCγ1 binds to EGF receptor and PDGF receptor via its src homology 2 (SH2) domain. Association of PLCγ1 to growth factor receptors leads to its phosphorylation by the receptor tyrosine kinase.

We have shown that in A431 cells EGFR is coimmunoprecipitated with a group of proteins recognized by antibodies to phosphoinositide-specific phospholipase Cy. These are 145-and 47-kDa proteins corresponding to phosphoinositide-specific phospholipase Cy and NCK respectively and unidentified 66-kDa protein. The association of phosphoinositide-specific phospholipase Cy1 and 66-kDa protein to EGF receptor was observed in A431 cells with or without EGF treatment. Trypsin peptide maps of these two proteins were similar so it can be suggested that 66-kDa protein is related to phosphoinositide-specific phospholipase Cy, maybe due to its proteolytic degradation.

EYE DEVELOPMENT IS AFFECTED BY CHOLINERGIC MOLECULES

We - 11

F. Morescalchi, C. Angelini, ^aG. Cimoli, ^cC. Falugi.

Institute of Comparative Anatomy, University, Genova, Italy;

^aNational Institute for Cancer Research, Genova, Italy.

Factors controlling growth of the central nervous system immediately after neurulation are mostly unknown. Cholinergic receptors, both muscarinic and nicotinic are widely represented in the nervous portion of the avian eye, and cholinergic molecules can also be found in developing lens, further, acetylcholine (ACh) has a trophic function during neurogenesis (Filogamo and Marchisio, *Neurosci. Res.* 4, 29, 1971). To verify the potential effects of cholinergic molecules on the eye growth, we used *in ovo* developing early chick embryos. Agonist and antagonist cholinomimetic drugs, ACh-competitive to cholinergic receptors, were applied by both "blind" injection in the yolk just beneath the blastodisc, and by windows in the egg shell. In this last case, drugs were injected into the yolk or just layed over the vitelline membrane covering embryos. Drugs were employed at different concentrations, ranging between 10 μM and 1 nM, and in different developmental stages: 1) during gastrulation; 2) during neurogenesis; 3) at the optic vesicle stage. The higher concentrations of drugs caused strong general anomalies, and death of embryos; 1 nM drugs showed a different effect: atropine (agonist of the G-protein-associate muscarinic receptor) strongly inhibited eye growth and pigmentation, and also the mesencephalic vesicle was smaller than in controls. Carbachol (agonist of the same receptor), on the contrary, showed an inhibitory effect when applied at the stage 1) and a growth-factor-like effect, when applied at further developmental stages. Furtherly, carbachol exhibited the eye growth factor-like effect also at early stages, when it was applied through the egg shell window on the vitelline membrane. Nicotine strongly inhibited eye development at all stages. The number of mitoses, the cholinesterase (ChE) activity, the muscarinic receptor expression, and the intracellular localization of protein kinase C were also affected by drug treatment.

EXPRESSION OF THE IGF-II/M6P RECEPTOR IN OVINE GRANULOSA CELLS *IN VITRO*

We - 12 P. Monget, C. Pisselet, D. Monniaux.

Station INRA de Physiologie de la Reproduction des Mammifères Domestiques, URA 1291, 37380 Nouzilly, France

In vivo in the ewe ovary, the IGF-II/M6P receptor is present at high levels in granulosa of atretic follicles. Conversely, very low levels are found in granulosa of healthy follicles (Teissier et al., 1994). The present study was conducted to understand the regulation of the expression of IGF-II/M6P receptor in ovine granulosa cells *in vitro*.

Ovaries from synchronized ewes were removed at Day 0 of the cycle (follicular phase) and granulosa cells were isolated from follicles 1-3 mm in diameter. Cells were seeded at different densities (from 5000 to 240000 cells/cm²) in tissue culture chamber slides and cultured for 48 hours in B2 medium containing fetal ovine serum (2%) with or without IGF-I (10 ng/ml). Serum was then withdrawn in half of the wells, and cultures were carried on for 24, 48 or 72 hours with or without IGF-I. At the end of the culture, medium was removed, IGF-II/M6P receptor was revealed on granulosa cells by both immunohistochemistry and autoradiography, and quantification was performed by image analysis. The number of cells per unit surface (cell density) was assessed by microscopic counting.

The number of IGF-II/M6P receptors per cell was inversely correlated with the cell density. Furthermore, after serum withdrawal, both the number of pyknotic granulosa cells and the number of IGF-II/M6P receptors per granulosa cell increased, as observed *in vivo* in atretic follicles. IGF-I increased both cell viability and cell density and decreased the number of IGF-II/M6P receptor per cell. The increase in IGF-II/M6P receptor after inducing cell death *in vitro* was not directly related to the decrease in cell density since 24 hours after serum withdrawal, both the number of IGF-II/M6P receptors and the cell density increased. To further investigate if the effect of cell density was related to cell-cell contact, granulosa cells were first cultured at low density for 48 hours in B2 medium with serum 2%, then cultures were carried on for 48 hours in B2 medium with or without serum, and in the presence of different concentrations of whole ovarian membranes. Incubation of cells with ovarian membranes significantly decreased the number of IGF-II/M6P receptors.

These results suggest that the expression of IGF-II/M6P receptor in ovine granulosa cells *in vitro* is regulated by both cell viability and cell-cell contact.

We-13 PRESENCE OF A 97 kDa PROTEIN IMMUNOREACTIVE TO TRANSFORMING GROWTH FACTOR- α (TGF α) ANTIBODIES IN THE COLON

M. Asbert, C. Diaz-Ruiz and
R. Pérez-Tomás.

Departament de Biología Celular i Anatomia Patológica, Facultat de Medicina, Universitat de Barcelona, Spain.

TGF α is a 8 kDa peptide homologous in structure and action to EGF. This growth factor is synthesized as part of a larger precursor, proTGF α of 22 kDa, an integral membrane glycoprotein, and it is released to extracellular media after cleavage by an elastase-like enzyme. TGF α is a potent mitogen for a variety of epithelial and mesenchymal cells. It has been detected in certain normal tissues and cultured cells suggesting a function in regulating normal cell proliferation and differentiation. Nevertheless proTGF α , as well as multiple higher molecular forms (18-68 kDa) are commonly expressed in many human tumors and tumor cell lines.

Cells from villi and crypts of both proximal and distal rat colon were isolated by a low temperature method. Lysates from rat intestinal epithelial cells and SW620, a colon carcinoma cell line, were subjected to tricine-SDS-PAGE system and transferred to Immobilon-P membranes. Western blot of rat colon showed a protein of apparent molecular weight of 97 kDa in proximal and distal crypts and proximal villi. In SW620 cell lines a protein with a similar molecular weight was also detected.

These results suggest that normal epithelial cells from rat colon and human tumor cell lines (SW620) synthesize a protein that could be a high molecular weight TGF α . The difference between this protein and proTGF α could be explained by a change in its glycosylation pattern.

We-15 RAS-DEPENDENT AND -INDEPENDENT REGULATION OF EXPRESSION AND PHOSPHORYLATION OF EARLY-RESPONSE GENE PRODUCTS IN NGF-STIMULATED PC12 CELLS.

N. Nusser¹, M. Pap¹, J. Szeberényi¹ & G.M. Cooper²

¹Department of Biology, University Medical School of Pécs, Pécs, Hungary; ²Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA

A variety of agents, including growth factors, second messengers, causes the rapid transcriptional activation of early-response genes (ERGs) in the PC12 pheochromocytoma cell line. This induction of several of ERG-coded mRNAs is accomplished via Ras-mediated signalling pathways. The present experiments were carried out to study whether the expression, synthesis and phosphorylation of three ERG-coded proteins (c-Fos, Zif268 and Nur77) stimulated by nerve growth factor (NGF) in PC12 cells are Ras-dependent or Ras-independent. PC12 subclones expressing different levels of a dominant negative Ha-Ras-protein (Ha-Ras-Asn17) were used in this investigation. Increased synthesis and phosphorylation of c-Fos and Zif268 proteins stimulated by NGF were detected both in control PC12 cells and in low expressor Ha-Ras-Asn17 sublines. In contrast, these processes were almost completely inhibited in high expressor Ha-Ras-Asn17 subclones. Similar studies on Nur77 indicated that both NGF-induced expression and phosphorylation of this protein are mediated by Ras-independent mechanisms. These results also suggest that NGF stimulation of expression and phosphorylation of these three ERG products are not sufficient to mediate late gene activation and neuronal differentiation in PC12 cells.

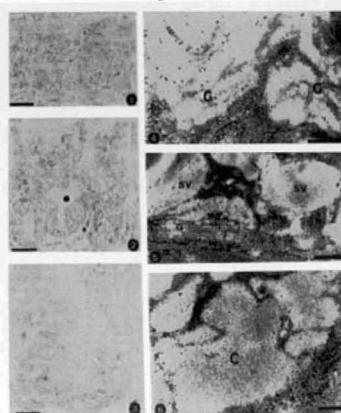
IMMUNOHISTOCHEMICAL LOCALIZATION OF TGF- α IN RAT COLON

We-14 R. Pérez-Tomás, M. Asbert and C. Diaz-Ruiz

Dept. Biología Celular. F. Medicina. Univ. de Barcelona. Spain. Fax: 34-3-4021896.

Transforming growth factor α (TGF- α) is a 50-amino acid polypeptide which has been related to cell proliferation and differentiation. Proximal and distal colon from fetal, newborn and adult rats were studied by immunohistochemical techniques using a monoclonal antibody against human and rat TGF- α . Immunoreactive TGF- α (IR-TGF- α) first appeared in distal colon at 18 days of gestation (Fig.1) when the proximal colon remained negative. At all ages studied, the staining for TGF- α at the base of the crypts in the distal colon showed a supranuclear pattern (Figs.2,3). At 22 days of gestation and until 9 days of postnatal development, the proximal colon is negative for TGF- α . From day 10 to 24 of postnatal development

IR-TGF- α cells with a cytoplasmic staining were confined to the lower half of the villi. Afterwards, cells at the crypts showed supranuclear immunostaining in the cis and trans cisternae of the Golgi apparatus (Figs.4,5,6) and cells in the surface epithelium a cytoplasmic immunoreaction in the polyribosomes. These results suggest a functional role for TGF- α in the establishment and maintenance of proliferation and differentiation during development.



We-16 EVIDENCE FOR THE EXPRESSION OF THE DOPAMINE D₂ RECEPTOR IN AtT20 MOUSE PITUITARY TUMOR CELLS

A. Winkler, W.-E. Siems, I. Putscher, M. F. Melzig

Res. Inst. of Molecular Pharmacology, 10315 Berlin, FRG

Two types of dopamine receptors mediate the effects of dopamine, the D₁ and D₂ receptor subtypes. D₂ receptors are the target for drugs used in the treatment of schizophrenia and parkinson disease. Additionally, there is experimental evidence that addiction to ethanol is realized through participation of the dopamine system. Furthermore, dopaminergic compounds can regulate the proopiomelanocortin (POMC) mRNA levels in rat pituitary. Interestingly, the POMC gene expression is decreased during chronic ethanol consumption, indicating a possible role of the D₂ receptor. Recent investigations have revealed an influence of dopamine derived Tetrahydroisoquinolines (TIQ's) on the POMC gene expression in the neuroendocrine cell line AtT20. Binding studies on AtT20 cells showed a low, but specific binding for the D₂ receptor. For this reason we expected a low D₂ mRNA content in these cells. In situ hybridization studies showed D₂ receptor mRNA only in individually cells in the anterior pituitary lobe without further characterization. We have used the sensitive RNase protection assay to detect D₂ mRNA in AtT20 cells. An 660bp fragment of the N-terminal region of the D₂ receptor was in vitro transcribed in the presence of radioactive-labelled UTP and used as a probe. Our studies revealed the existence of a D₂ receptor mRNA in AtT20 cells providing further evidence for a possible role of this D₂ receptor in pharmacological effects.

TGF- α -LIKE PROTEIN IN THE CHICKEN KIDNEY

C. Díaz-Ruiz, M. Asbert and
R. Pérez-Tomás.

We-17

Departament de Biologia Cel.lular i
Anatomia Patològica, Facultat de
Medicina. Universitat de Barcelona,
Spain.

Transforming Growth Factor- α (TGF- α) is a polypeptide related to EGF. Both bind to EGF-Receptor (EGF-R) to carry out their function. We have identified a TGF- α -like protein in the chicken kidney by immunohistochemistry and Western blot analysis. Chicken kidneys from embryos and adult animals were fixed in Bouin, dehydrated, embedded in paraffin and sectioned. We used a monoclonal anti-human TGF- α (epitope 34-50) and the immunoperoxidase technique, and counterstained sections with alcian blue or PAS. The immunoreactive cells for TGF- α were localized in the distal tubules of the nephrons (alcian blue and PAS negative tubules) and showed an alternating pattern of distribution in the tubules. Scarred cells of the collecting tubules were also immunostained. Immunoreactivity was mainly located in the cytoplasm. Kidneys from postnatal chickens and rats were homogenized. Membranous, membrane-extracted and cytosolic fractions were collected and proteins were separated by the tricine-SDS-PAGE system and transferred to immobilon P membranes. Using two monoclonal anti-TGF- α antibodies we detected a protein of apparent molecular weight of 14 and 31 kDa in the cytosolic fraction and also of 45 kDa in the membrane-extracted fraction. These results suggest that a TGF- α -like protein is specifically expressed in some cells of the distal tubules and may be involved in the differentiation mechanisms of those cells.

We-19

ACTION OF AN ANIMAL TISSUE EXTRACT ON
IN VITRO CELL GROWTH: PRELIMINARY DATA

DOINA PETRASINCU a), D. STANIOAE b)

a) Tissue Culture Department, Cantacuzino Institute, Bucharest, Romania; b) Biochemistry Department, Research Institute for Wastewater, Bucharest, Romania.

The healing of some inflammatory diseases, which affect particularly tissues like skin or cornea, implies firstly the proliferation of a new normal tissue. The use of growth factors for improving the cell growth seems to be potentially beneficial.

In the present work, we study the effect of an animal tissue extract - named M1 - on the "in vitro" cell growth. The "in vitro" growth dynamics of human diploid cells treated with M1 extract shows a high and concentration dependent stimulation of the cell growth, measured by specific parameters (cell density at 24 hrs. intervals, cell density at confluence, population doubling time).

The effect of M1 extract on cell morphological structures, like cytoskeleton, implied in the "in vitro" cell growth, as well as on the proliferation of different cell types in culture remain to be investigated.

We-18

IS SEROTONIN A NOVEL AUTOCRINE GROWTH FACTOR FOR HUMAN SMALL CELL LUNG CARCINOMA CELLS?

M.G.Caltanego^a, A.Codignola^b, E.Sher^b,
F.Clementi^{a,b} and L.M.Vicentini^a

^aDepartment of Pharmacology and ^bCNR Center of Cytopharmacology, University of Milan, Milan, Italy

Small cell lung carcinoma (SCLC) is an aggressive human tumor often associated with tobacco smoking. It has been shown that SCLC cells contain peptides for some of which a role as autocrine growth factor has been suggested.

We found that 5-HT is contained in two SCLC cell lines tested (GLC8 and NCI-N-592) and is released in the culture medium. When added to the culture medium 5-HT was a mitogen for SCLC cells. Moreover, when cells were cultivated at higher density, 5-HT antagonists were able to inhibit basal cell proliferation. The use of specific agonists and antagonists of the multiple 5-HT receptor types indicated that the 5-HT_{1D} receptor is at least in part responsible for the mitogenic effect of 5-HT. The receptors belonging to the 5-HT₁ family are negatively coupled to adenylate cyclase. In SCLC cell membrane preparations 5-HT inhibited by 17% the forskolin-stimulated adenylate cyclase activity.

We also found that nicotine, whose receptors of the neuronal type are expressed in these cells, stimulated 5-HT release from SCLC cells as well as cell proliferation. The dose-response curves for the two nicotinic effects corresponded closely.

In conclusion, the data presented indicate that 5-HT should be added to the list of autocrine growth factors contained and released by SCLC cells.

We-20

Expression of FGFR-3 mRNA in the central nervous system and in the mesenchymal condensation sites during amphibian morphogenesis

V. Fromentoux, D. L. Shi and J. C. Boucalt

Laboratoire de Biologie Expérimentale, URA-CNRS 1135, Université P. et M. Curie, 9 quai Saint-Bernard, 75005 Paris, France

Fibroblast growth factors (FGFs) and their receptors (FGFRs) are involved in the control of embryogenesis. We have isolated an amphibian homolog of the human FGFR-3, which binds both aFGF and bFGF when expressed in *Xenopus* oocytes. The amphibian FGFR-3 is a maternally derived mRNA. Low levels of expression were detected during cleavage and gastrula stages, however, a significant increase of the mRNA was observed at the beginning of neurulation. RNase protection analysis showed that FGFR-3 mRNA was mainly localized to the ectoderm of the early gastrula and then shifted to the embryonic neural tissues, while adult brain had decreased levels of expression. In situ hybridization revealed strong expression in the germinal epithelium of the embryonic brain (especially the diencephalon and rhombencephalon), in the lens and in the cranial ganglia. The epithelium of the developing gut like the pharynx and esophagus also expressed prominently the mRNA. In addition, FGFR-3 was found to be expressed most strongly in the mesenchymal condensation sites of the branchial arches and head mesenchyme. These observations suggest that the amphibian FGFR-3 may play a role in neural development and in the process of mesenchymal condensation.

We - 21

TGF β effect on growth and differentiation of rabbit tracheal outgrowths in primary culture.
S. Boland, E. Boisvieux-Ulrich, A. Baeza, F. Marano.
Laboratoire de Cyrophysiologie et de Toxicologie Cellulaire, Université Paris VII, Paris, France.

Transforming growth factor TGF β is a multifunctional regulator of growth and differentiation. While it stimulates proliferation of cells of mesenchymal origin, it inhibits growth of various epithelial cell types. It is a potent inducer of squamous metaplastic differentiation in normal tracheal cells in vitro culture, but transformed tracheal cells lines have altered growth factor sensitivity. Squamous metaplasia of tracheal epithelium occurs *in vivo* after various injury, as a repair mechanism, characterized by a multistep transformation including first a proliferative stage, followed by the establishment of squamous phenotype with a strong modification in the expression of cytokeratins as in terminal epidermal differentiation. It is also considered a preneoplastic lesion.

Primary culture of rabbit tracheal cells according to the method of explants results in formation of an outgrowth by migration and proliferation of cells of the explant. Squamous metaplasia can be reproduced when cells are grown on a thick collagen gel. TGF β treatment induces a dose-dependent increase in outgrowth surface. However cell proliferation is inhibited by TGF β as measured after BrdU incorporation and cell counting. Cell-cell adhesion and cell-extracellular matrix adhesion appear affected. TGF β regulation of cell migration and cell shape is investigated in respect with modifications of expression or organization of cytoskeletal components, such as actin and cytokeratins.

We - 23

TRANSBILAYER REDISTRIBUTION OF PLATELET PHOSPHOLIPIDS (PL) IN PATIENTS WITH PSYCHOSES
A. Struneká^a, D. Řípová^b, I. Farská^b

^aDepartment of Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 7, Prague 2;

^bLaboratory of Biochemistry, Prague Psychiatric Center, Ústavní 91, Prague 8, Czech Republic

Despite the intensive research in psychoses, biochemical markers have not been found. We have investigated the changes in the asymmetrical distribution of PL in platelets isolated from the blood of schizophrenic and manio-depressive patients. The lipophilic fluorescence probe Merocyanine 540 has been used as a tool for rapid screening of transbilayer redistribution of PL. Changes in the fluorescence after platelet activation with calcium ionophore A 23 187 were measured using flow cytometry. We have found the difference in the distribution of aminophospholipids in the group of schizophrenic patients ($n = 11$, D/S(N), $p < 0,05$) as compared with the group of healthy donors ($n = 10$), while no significant difference was found between the manio-depressive ($n = 10$) and the control group.

We - 22

EFFECT OF CHLORPROMAZINE (CPZ) ON PHOSPHOINOSITIDE-SIGNALLING SYSTEM IN HUMAN PLATELETS

D. Řípová^a, V. Němcová^a, C. Höschl^a, A. Struneká^b

^aLaboratory of Biochemistry, Prague Psychiatric Center, Ústavní 91, Prague 8; ^bDepartment of Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 7, Prague 2, Czech Republic

Some of the varied pharmacological effects of the amphiphilic cationic drug chlorpromazine could be ascribed to its interaction with phospholipids and proteins involved in the phosphoinositide-signalling pathway. We have studied the effect of CPZ on the 32 P incorporation in vitro into phosphoinositides (PI, PIP and PIP₂) and phosphatidic acid (PA) in human platelets. The cytosolic level of Ca²⁺ was measured using fluorescence probe Fluo-3. 5×10^{-4} M CPZ decreases 32 P incorporation into PI and PIP₂ to 44 and 22% of control values, respectively. 5×10^{-5} M CPZ evokes the increase of 32 P incorporation into PA and PI (179 and 195% of control values). 32 P incorporation into PIP₂ is not affected. CPZ in all concentrations studied decreases the level of cytosolic Ca²⁺. Inhibition of protein phosphorylation was observed.

We - 24

MECHANICAL INDUCTION OF CALCIUM SIGNALING VIA INTEGRINS IN HEPATOCYTES
B. Nebe, J. Rychly, W. Bohn¹, A. Knopp

Dept. of Internal Medicine of the University, Rostock, Germany;
¹Heinrich-Pette-Institute for Experimental Virology and Immunology, Hamburg, Germany.

Integrins are heterodimeric adhesion receptors consisting of α and β chains which mediate signals in intercellular interaction and between cells and extracellular matrix. In the immortalized hepatocyte cell line mHepR1 we investigated intracellular calcium changes induced by mechanically stimulating the integrin subunits as a parameter for signal transduction.

For calcium measurements hepatocytes were loaded with Fluo-3 and after stimulating the integrins the fluorescence was detected by flow cytometry. We found that incubation with monoclonal anti-integrin antibodies for 15 min., washing the cells and a subsequent mechanical stimulation led to an increase of the intracellular Ca²⁺. The calcium signals were due to a release from intracellular reservoirs. A6, a receptor for laminin provoked a higher signal than $\alpha 2$ and $\beta 1$. No Ca²⁺-signal was obtained after adding antibodies and immediate measurement of intracellular calcium.

In parallel experiments with monolayers of hepatocytes we analyzed the physical association between integrin and cytoskeleton by immunocytochemistry.

The experiments revealed the relevance of integrins in signal transduction induced by mechanical stimulation in hepatocytes. The relationship between Ca²⁺-signal and the linkage of integrins with the cytoskeleton is discussed. Quantitative differences in calcium signals may reflect the functional significance of an integrin subunit.

We-25 RB3: A NEW MEMBER OF THE STATHMIN FAMILY HIGHLY EXPRESSED IN BRAIN
 S. Ozon, A. Maucuer and A. Sobel.
 INSERM U153-CNRS ERS64, Paris, France

Stathmin (also designated p19, oncoprotein 18 or prosolin) is a 19 kDa cytoplasmic phosphoprotein, presumably a general intracellular relay integrating diverse signal transduction pathways (TIBS 16, 301). Its expression is regulated during early and late embryonic and postnatal development as well as in relation with cell proliferation and differentiation (see abstract by Balogh and Sobel). The sequence of stathmin is well conserved throughout vertebrate evolution. Two proteins sharing a stathmin-like domain and an additional N-terminal region have been identified: the rat protein SCG10 specifically related to neuronal differentiation and XB3, a putative *xenopus* protein whose cDNA was cloned by homology with stathmin.

In this study, we searched new members of the stathmin family in mammals; a rat striatum cDNA library was thus used for PCR amplification of stathmin-related sequences. We characterized a rat cDNA encoding a protein sharing 70% aminoacid identity with rat stathmin in their common domains, and 85% identity over the entire sequence of *xenopus* XB3: we thus designate RB3 the rat protein encoded by the isolated cDNA. Northern blot analysis with a specific probe to RB3 cDNA showed the presence of a 1 kb mRNA highly expressed in rat brain. We raised an antibody against a synthetic peptide corresponding to part of the RB3 sequence. Immunoblotting revealed a 35 kDa protein with a pI around 7, abundant in rat brain homogenate and in PC12 cells.

The biochemical and biological characterisation of this new member of the stathmin family will help to investigate the general function of stathmin and the specificity of the related proteins.

We-27 INTRANUCLEAR PIP₂ POOL IS SELECTIVELY AFFECTED BY AGONISTS IN DIFFERENT CELL TYPES

N. Zini^a, S. Marmiroli^a, A. Ognibene^b, I. Faenza^b, A. Valmori^a, G. Mazzotti^c and N.M. Maraldi^a

^a Ist. Citomorfologia N. e P. del C.N.R., ^b Lab. Biologia Cellulare e Microscopia Elettronica, c/o IOR, via di Barbiano 1/10, Bologna, Italy; ^c Ist. di Anatomia Umana, Università di Bologna, Italy.

The breakdown of polyphosphoinositides, recently reported to occur also at the nuclear level (1), is modulated by specific agonists. Swiss 3T3 fibroblasts mitogenically stimulated by IGF-1 (1), Friend cells induced to erythroid differentiation by DMSO (2) and human osteosarcoma SaOS-2 cells treated with Interleukin 1 α (3) were utilized for determining quantitative variations of nuclear PIP₂ breakdown by immunocytochemical electron microscopy utilizing a MoAb against PIP₂ (4). While no significative variations occur at the cytoplasm level, the nuclear amount of PIP₂ found in the heterochromatin domains is decreased in IGF-1 - treated Swiss 3T3 cells and in Interleukin 1 α -treated SaOs-2 cells, and increased in DMSO-differentiated Friend cells. These data agree with the variations of PI-PLC β activity found in each cell type treated with specific agonist. Mitogenic and differentiating stimuli appear to affect in a opposite manner the phosphoinositide signaling system operating at the nuclear level.

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We-26 EFFECT OF ARGININE-VASOPRESSIN ON PHOSPHOINOSITIDE METABOLISM OF RAT PINEAL GLAND

R. Novotná^a, Z. Zelenková^a, R. Krulík^b and I. Novotný^a

^a Department of Animal Physiology, Charles University, Prague, ^b Psychiatric Research Unit, Charles University, Prague

Although much is known about sympathetic innervation of rat pineal gland, the central innervation which contains arginine-vasopressin (AVP) fibres is less documented. The aim of the present study was to find out, how AVP mediates its effect on pineal gland signalling system.

Individual rat pineal glands were incubated in conventional medium with 50 uC ³²P orthophosphate and AVP. ³²P labelling of phosphoinositides was determined after extraction and separation of phospholipids. AVP (0.01 mM) increased ³²P labelling into phosphatidylinositol 3 times and into PIP₂ 5 times compared to the control. Labelling of other phospholipids was not affected. V₁ receptor antagonist blocked labelling of PIP₂. Activity of cAMP dependent protein kinase and phosphorylation of proteins was not affected by AVP. It is suggested that phosphoinositides play a role in signal transduction in rat pineal gland in response to AVP and not a signalling system involving cAMP.

We-28 PI-PLC δ IS GREATLY INCREASED DURING PC12 NEURAL DIFFERENTIATION

L.M. Neri^{*a}, D. Milani^{*}, L. Bertolaso^{*}, M. Marchisio*, S. Capitani*

^a Ist. Anatomia Umana, Univ Ferrara, Fossato Mortara 66, Ferrara, Italy; ^b Ist. Citomorfologia Normale e Patologica, CNR, Bologna, Italy.

Among the several enzymes involved in the phosphoinositide metabolism, phosphatidylinositol-specific phospholipase C (PI-PLC) β , γ and δ have been shown to be present in rat pheochromocytoma PC12 cells (1) and selectively localized in the nucleus (β isoform), in the cytoplasm (δ isoform) or in both cell compartments (γ isoform). Changes in PI-PLC isoforms presence and distribution were analyzed after Nerve Growth Factor (NGF) administration. Within three days of NGF exposure cells showed neural processes extended to twice the diameter of the cell body; the γ isoform was concentrated near the nucleus, while the immunoreactivity of the β isoform remained constant in the nucleus and the δ isoform was already increased. This distribution was modified after 14 days of NGF treatment, i.e. after neural differentiation of the cells. Fully differentiated cells displayed an intense PI-PLC δ immunolabelling covering the whole cell body, also including neurites. In the same cells, PI-PLC β immunostaining left only few traces in the nucleus and PI-PLC γ immunoreactivity was only slightly detectable in cell bodies, nuclei and neurites (2). These results suggest that a significantly higher presence of PI-PLC δ is associated with the neural differentiation, the decrease of cell proliferation and the selective down regulation of PI-PLC β and γ in PC12 cells.

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We-29 THE ACTIVITY OF TRANSDUCING CELLULAR SIGNALS IN HUMAN LEUKEMIAS
 J. Rzymowska
 Department of Cell Biology,
 University of M. Skłodowska-Curie, Lublin, Poland

The ectoenzym gamma glutamyltransferase (GGT) (EC: 2.3.2.1) a second messenger generating enzyme activity on the cytoplasmic membrane was biochemically analyzed in leukemic cells and serum from patients with acute lymphoblastic and myeloid leukemias. Mononuclear cells were separated by mean of Ficoll-hypaque gradient centrifugation. The mean values of GGT activity on serum were significantly vary among the two groups of leukemias. The values at patients with acute lymphoblastic leukemia were found lower than these at patients with acute myeloid leukemias. The mean activity - 0.594 IU/mg protein was measured in the patients with acute lymphoblastic leukemias (ALL), while the higher - 0.956 IU/mg protein was found in acute myeloid leukemias (AML) on serum. The GGT activity at control of mononuclear cells was higher than this at ALL and AML patients. There didn't difference between values of GGT activity at ALL and AML patients. The high levels of GGT activity on serum provide higher concentrations on the outside morphonuclear cells than on the inside these cells. The low GGT activity might be characteristic of the leukemic transformation independent however of the blast origin. The level of GGT may be inversely proportional to the degree of myeloid differentiation, as was established on morphologic, cytochemical and immune criteria. Leukemic cells of myeloid lineage these functional constituents located on the external lipid leaflet of the plasma membrane were independently affected by the neoplastic process. An altered composition and organization of functional and structural constituents in the plasma membrane of myeloid cells is a peculiar trait describe in other leukemic processes.

We-31 LOCALIZATION OF SPERM PROTEINS DURING CAPACITATION AND ACROSOME REACTION
 J. Pěkníková^a, J. Moosa^a, J. Čapková^a,
 J. Sršen^b

^aInstitute of Molecular Genetics, Prague, Czech Republic; ^bInstitute of Physiology and Genetics of Animals, Libeňov, Czech Republic

Three monoclonal antibodies that stained acrosin of 55, 53, 45 and 38 kDa (ACR.2), acrosomal protein of 28 kDa (ACR.4) were used for immunostaining and immunoblotting analysis of boar spermatozoa before and during capacitation and ionophore-induced acrosome reaction. Two protein (acrosin and the 28 kDa protein) showed similar changes in the acrosome state during capacitation and induced acrosome reaction. For the detection of these proteins immunolectron microscopy was also used. The immunofluorescence test revealed that most spermatozoa lost acrosin and 25 kDa protein after incubation with ionophore A 32187. The lost protein was detected in the culture medium by immunoblotting.

Since the release of the acrosin and 28 kDa antigen from sperm cells is strongly correlated, we concluded that the 28 kDa acrosomal protein may help to keep the acrosomal matrix integrity delaying acrosin liberation from acrosome reacted cells. The 17 kDa protein (ACR.3) showed a different pattern of labelling. The changes in relocation of specific proteins during capacitation and acrosome reaction support a concept of physiological preparation of spermatozoa for sperm-egg interaction.

We-30 CHARACTERIZATION OF AT₂ RECEPTOR EXPRESSION IN NIH 3T3 FIBROBLAST CELL LINE
 S. Zorad^a, N. Xu^c, F.M.J. Heemskerk^b,
 S.J. Gutkind^c and J.M. Saavedra^b

^aInstitute of Experimental Endocrinology, SAS, Bratislava, Slovakia, ^bSection on Pharmacology, LCS, National Institute of Mental Health and ^cLaboratory of Cellular Development and Oncology, National Institute of Dental Research, Bethesda, Maryland, USA.

The octapeptide hormone, angiotensin II (Ang II) binds to at least two subtypes of receptors, termed as AT₁ and AT₂. The AT₁ belongs to the family of G-protein-coupled receptors. The recently cloned AT₂ seems to be a member of unique class of seven-transmembrane receptors lacking G protein coupling. Until recently AT₂ has no defined cellular function.

We found high expression of Ang II receptors and angiotensin converting enzyme (ACE) activity in confluent NIH 3T3 fibroblast cell line seeded at 4×10^4 cells/plate concentration. The receptors are exclusively of AT₂ subtype as determined by competition studies using selective subtype specific ligands, PD 123177, CGP 42112A and Losartan and by insensitivity to dithiothreitol and GTPγS. AT₂ receptor expression was very low in sparse culture and was totally missing in culture seeded at density higher than 10^5 cells/plate. The AT₂ receptors were also present in c-ras transformed NIH 3T3 cells lacking contact inhibition. AT₂ expression in c-ras transformed cells was independent on the seeding conditions. Our results imply the possible relation of the Ang II, ACE and AT₂ receptors to the cell growth and/or cell - cell contact.

We-32 THE EFFECT OF PRETREATMENT WITH ETHYLENE GLYCOL ON INTERCELLULAR GAP JUNCTIONAL COMMUNICATION

J. Vitek
 Research Institute of Child Health,
 Laboratory of Cell Biology, Brno, Czech Rep.

The Chinese hamster V79-4 cell line and/or its thioguanine resistant derivative had been pretreated with ethylene glycol (EG) for 72, 48 or 24hrs (medium with the drug was renewed every 24hrs) and afterwards the Intercellular Gap Junctional Communication (IGJC) was detected by our modification of Metabolic Cooperation Assay.
RESULTS:

1. pretreatment for 24hrs led to the inhibition of IGJC, but lower than that of the treatment with EG during the MC-assay itself.

2. pretreatment for 24hrs of only one partner (donor or recipient) was sufficient for the significant inhibition of IGJC, although, in this case, the effect was lower.

3. longer pretreatments (48 or 72hrs, with the medium renewed every 24hrs) led to the loss of the inhibition of IGJC.

CONCLUSION:

The longer was the EG-pretreatment the lower was the inhibition of IGJC.
 The meaning of this finding will be discussed.

We-33 Interactions of Opioids with G-proteins in Rat Brain Membranes

G. Fábián, M. Szikszay^a, M. Szűcs

Inst. of Biochem., Biol. Res. Center, Hung. Acad. Sci. and ^aDept. of Physiol., Szent-Györgyi A. Medic. Univ., Szeged, Hungary
In the transduction of opioid signals the pertussis toxin-sensitive G-proteins, G_i and G_o have been implicated.

Previously we detected specific opioid binding sites not only in the synaptic plasma membrane (SPM) fraction, but about 30% of the total pool was found in the so-called microsomal (MI) fraction. Previous binding experiments indicated that while opioid receptors act via G-proteins in the SPM, they might be uncoupled in the MI. In the present work we have studied the G-protein content of these fractions by immunoblots with specific antisera against α -subunits of appropriate G-proteins. The main types, G_i , G_o , G_s were assessed in both SPM and MI. This assay as well as GTP- γ S binding, pertussis toxin catalysed ADP-ribosylation and photoaffinity labelling experiments solved that chronic morphine treatment of rats resulted in elevated level of G-proteins in both fractions. This might be the outcome of compensatory mechanisms of the cells to overcome the consequences of receptor down-regulation which occurs after prolonged agonist exposure.

This work was supported by OTKA-895 research grant and UNESCO SC/RP 201277.3 fellowship.

We-34 CHRONIC EXPOSURE OF DEVELOPING CHICK NEURONAL CELLS TO LOW DOSES OF KAPPA-OPPIOID AGONISTS LEADS TO ACCELERATED DIFFERENTIATION AND RECEPTOR DOWN-REGULATION

K. Maderspach, B. Csépán, L. Bajenaru and P. Tóth
Inst. Biochemistry, Biological Res. CTR., Hungarian Academy of Sciences, Szeged, Hungary.

It is well documented that external signals play regulatory role in early development of the nervous system. Recent data suggest that endogenous opioids, especially those of kappa-type, may also function as developmental signals. The primary cell culture provides an excellent tool to study this question since morphological and biochemical changes can be followed under controlled conditions.

Primary cultures were established from 7-day-old chick embryo forebrains on poly-lysine to get differentiating neurons. Opioid agonists as bremazocine (10^{-7} , 10^{-8} M), dynorphin (10^{-7} M), U-50,488 (10^{-6} M), (all kappa-type), morphine (10^{-6} M), (μ -type), and antagonists as norbinaltorphimine (10^{-7} , 10^{-8} M), (kappa-type) and naloxone (10^{-5} M), (non-selective), alone or in combination with agonists were added chronically to cultures between the cultivation days 1-4. Similarly, a monoclonal antibody (mAb KA8, IgG1,k) specifically recognizing the kappa-opioid receptor (Maderspach et al. J. Neurochem. 1991, 56, 1897) was added chronically into the culture medium.

Kappa-type agonists bremazocine and dynorphin and mAb KA8, in concentration dependent manner exerted trophic effect on neuronal morphological differentiation and caused significant down regulation of the kappa-opioid receptors. Reversion of this effect by antagonists was not complete. These results support that endogenous kappa-opioid agonists may regulate among others the neuronal differentiation through specific receptors.

We-35 INHIBITION OF CARDIAC SARCOLEMMAL Na^+/Ca^{2+} EXCHANGER BY PHOSPHORYLATED GLYCEROPHOSPHOINOSITOLS

S. Luciani, M. Antonini, S. Bova, G. Cargnelli, F. Cusinato, P. Debetto, L. Trevisi, R. Varotto

Department of Pharmacology, University of Padova, Padova, Italy

Cardiac Na^+/Ca^{2+} exchanger catalyzes the countertransport of Na^+ for Ca^{2+} across the sarcolemmal membrane and, owing to its dominant function in Ca^{2+} efflux, plays a main role in the beat to beat regulation of heart contraction.

Acidic phospholipids such as phosphatidylserine¹ have been demonstrated to be physiologically relevant regulating factors of the Na^+/Ca^{2+} exchanger. More recently it has been shown that polyphosphoinositides² increase Na^+/Ca^{2+} exchange activity.

In order to evaluate the relevance of the fatty acids or lipid head groups in the effect of polyphosphoinositides, the deacylation products of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-diphosphate (PIP₂), glycerophosphatidylinositol-4-phosphate (GroPins-4-P) and glycerophatidyl-inositol-4,5-diphosphate (GroPins 4,5-P), respectively, have been tested in cardiac sarcolemmal vesicles Na^+/Ca^{2+} exchange activity.

It was found that GroPins-4-P and GroPins-4,5-P are powerful inhibitors (IC_{50} : 5-10 μ M) of Na^+/Ca^{2+} exchange activity. The data suggest that in cardiac cells, the phosphoinositide metabolites GroPins-4-P and GroPins-4,5-P produced from deacylation of PIP and PIP₂ by activated phospholipase A₂ can modulate intracellular Ca^{2+} concentration by inhibiting the Na^+/Ca^{2+} exchanger.

1. S. Luciani, Biochim Biophys Acta, 772: 127-134, 1984.
2. S. Luciani, S. Bova, G. Cargnelli, F. Cusinato, P. Debetto, Ann. N.Y. Acad. Sci., 639: 156-165, 1991.

We-36 STIMULATION OF CACO-2 CELLS WITH VIP SHOWS AN EFFECT ON SEVERAL LOW MOLECULAR WEIGHT GTP-BINDING PROTEINS

R. Gerhard, J. Stein, S. Zeuzem and W.F. Caspary

Laboratory of Gastroenterology, 2. Medical Department, Johann Wolfgang Goethe-University, Frankfurt/Main, Germany

In the present study we investigated the influence of VIP on GTP-binding to low molecular weight G-proteins in the colon carcinoma cell line CaCo-2. Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum for 4 weeks until total differentiation and polarisation. Cells were then held in serum-free AIM-V supplemented with 1% glutamin for 24 h. VIP was added to a final concentration of 10^{-7} M and cells were incubated for 15 min. Incubation was stopped by washing cells with ice-cold mannitol buffer. After homogenization, plasma membranes were removed by differential centrifugation. On the remaining cytosolic fraction, containing transport vesicles and low density microsomes, two-dimensional electrophoresis and protein transfer on nitrocellulose were performed. Low molecular weight G-proteins were detected by [α ³²P]GTP-overlay and following autoradiography. VIP-stimulated cells showed an increased GTP-binding to a 27 kDa protein with isoelectric point of pH 5.6, and to two 25 kDa proteins with isoelectric points of pH 7.2 and 7.3. Moreover GTP-binding to a 25 kDa protein processing a pH of 5.3 and also to 27 kDa proteins at a pH of 5.2 and 5.4 were decreased. These findings indicate, that signalling of adenylate cyclase activating agonists involves several cytosolic low molecular weight G-proteins.

We-37 DOSE-DEPENDENT INHIBITION OF MYOGENIC RHYTHMIC CONTRACTIONS OF HUMAN MYOMETRIUM BY PHOSPHATIDIC ACID
C.Vidulescu, O.C.Trițan and L.M.Popescu
Department of Cell Biology and Histology,

"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

Phosphatidic acid (PA) is a natural product of phosphatidylcholine breakdown by phospholipase D (PLD) in smooth muscle cell membrane. PA may also result from diacylglycerol (DAG) phosphorylation by DAG kinase and may be the substrate of PA phosphohydrolase (PAP) that converts PA to DAG.

Using human myometrial smooth muscle strips *in vitro* we found that exogenously added PA (20 - 200 μ M) inhibited dose-dependently the amplitude and frequency of myogenic rhythmic contractions. Synthetic dipalmitoyl-PA had a similar, but weaker effect, showing that PA activity depends on its fatty acyl composition. Exogenous PLD (3 - 10 U/ml) also inhibited myogenic rhythmic contractions to a lesser extent.

In order to investigate the endogenous sources of PA we used 1,2-di-octanoyl ethylene glycol, an inhibitor of DAG kinase, which had no significant effect. In contrast, propranolol (40 - 250 μ M), an inhibitor of PAP, had a marked inhibitory effect on myogenic rhythmic contractions. Inhibitory anti-PLD antibodies (250 μ g/ml) augmented the frequency and finally the basal tone of myogenic rhythmic contractions.

These data indicate PA as a possible candidate for the design of a tocolytic agent and PLD as a key enzyme in the mechanism of myogenic rhythmic contractions and relaxations of human myometrium.

We-39 "PRO-INFLAMMATORY" EFFECT OF THE PARASYMPATHETIC SYSTEM ON THE ACTIVITY OF HUMAN NEUTROPHILS, MAST CELLS AND RAT LYMPHOCYTES

Gajewski M.^{*}, Laskowska-Bożek H.^{**}, Maślinski S.^{**}, Ryzewski J.^{***}. Dept. of Biochemistry,^{**}
Dept. of Pathophysiology, Institute of Rheumatology, Warsaw, Poland.
Metabolism of phagocytic cells may be substantially modified by neurotransmitters of autonomic system. Human peripheral neutrophils were studied to assess the effect of muscarinic cholinergic agonist (carbachol) on neutrophils luminol-enhanced chemiluminescence (CL). Carbachol was ineffective when resting neutrophils were tested. Not expected increase in CL of zymosan-activated neutrophils was observed following carbachol treatment. This increase was prevented by atropine, a muscarinic antagonist. Stimulation of rat lymph nodes lymphocytes with ConA resulted in a fall in intracellular ATP and increase in muscarinic binding. We have also shown, that the ability of cholinergic agonist to augment the effector functions of lymphocytes is directly related to the magnitude of muscarinic binding. The existence of muscarinic receptors on the human mast cells has been recently suggested. We showed that ATP released from stimulated mast cells is enhanced by cholinergic stimulation.

We-38 ACTIVATION OF HUMAN NEUTROPHILS BY DIFFERENT RECEPTORS
V.Ciălău and C.M.Tzigaret

Department of Cell Biology and Histology,
"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

The signal transduction pathways leading to neutrophil activation are poorly understood. G proteins, phospholipases C and D (PLC; PLD) systems and protein kinases seem to be involved in neutrophil activation.

We have studied neutrophil activation in two assay systems: stimulation with opsonized zymosan which interacts with Fc receptors and stimulation with fMLP, a chemoattractant peptide that binds to CR3 receptor.

Neutrophils were separated from normal human peripheral blood by density gradient centrifugation (SEPCEL, Babes Institute, Bucharest) and hypotonic lysis of erythrocytes. The cells were incubated in HEPES-buffered RPMI 1640 medium with 1 μ M dihydrorhodamine 123 (Molecular Probes, USA) a fluorochrome specific for oxidative burst activity. The samples were then stimulated with opsonized zymosan (1 mg/ml) or fMLP (0.1 μ M). For modulation studies, the samples were treated with anti-PLC or anti-PLD antibodies (500 μ g/ml), phorbol 12-myristate 13-acetate (150 nM) and ionomycin.

Data were analyzed on a FACStar Plus flow cytometer (Becton Dickinson, USA) by measuring the morphological and fluorescent parameters of the cells.

These results show that neutrophil activation by opsonized zymosan or fMLP occurs through different pathways and that anti-PLC or anti-PLD antibodies are able to inhibit signaling transduction in neutrophils.

We-40 Agonist (carbachol) induced transfer of Gq&G11 alpha proteins and of M1 muscarinic acetylcholine receptors from plasma membranes to a light vesicular (endosome) membrane fraction. The first step in down-regulation pathway?
P. Svoboda and G. Milligan

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; Institute of Physiology, Czech Academy of Sciences, Videnska 1083, Praha 4 - Krc, Czech Republic

A CHO cell line expressing high levels of an acetylcholine muscarinic receptor (human type 1, HM1) undergo a substantial agonist-specific down-regulation of both HM1 receptors and Gq&G11 proteins which is accompanied by a desensitization of phospholipase C response. This down-regulation phenomenon was analysed to identify a possible agonist-effect on subcellular distribution of Gq&G11 alpha proteins and of HM1 receptors. The plasma membrane as well as non-plasma membrane pools of Gq&G11 alpha proteins and of HM1 receptors were separated using sucrose density centrifugation and analysed after short-term (5-60 min) and long-term (16 hours) agonist treatment.

The short-term (30 min) incubation with carbachol (1 mM) induced a simultaneous transfer of both Gq&G11 proteins and HM1 receptors from plasma membranes to low-density membranes (light vesicles, endosomes) distinct from plasma membranes. The total number of receptors and of Gq&G11 proteins per cell remained unchanged under these conditions. Surprisingly, a similar transfer was manifested for Gs alpha protein. The plasma membrane as well as low-density (light-vesicle) membrane pools of Gs alpha subunit were unaffected by further incubation with carbachol (up to 16 hours), while the HM1 receptors and Gq&G11 proteins were down-regulated to 25% and 40% respectively when compared with control values in both membrane compartments.

The data support the idea that down-regulation of the HM1 receptor is represented by two subsequent steps. The first step is a transfer (internalization) of signal transmission components from plasma membranes to non-plasma membrane vesicles. The second step is represented by an agonist-specific down-regulation pathway. Both HM1 receptors and Gq&G11 proteins seem to follow similar sequestration and down-regulation patterns.

We-41 COEXPRESSION OF MIDDLE- AND LONG-WAVELENGTH SENSITIVE PHOTORECEPTOR MOLECULES IN ONE RETINAL CONE CELL

P. Röhlich^a, Th. van Veen^b, and Á. Szél^{a,b}

^aLaboratory I of Electron Microscopy and 2nd Dept. of Anatomy, Semmelweis University of Medicine, H-1450 Budapest, Hungary, and ^bDept. of Zoology, Göteborg University, S-413 90 Göteborg, Sweden

Color vision requires a retinal photoreceptor mosaic in which each visual cell is maximally sensitive to a certain wavelength of light. This assumes that each cone cell contains one, and only one type of photoreceptor molecule (visual pigment). Here we furnish evidence that cones can simultaneously express two different visual pigments in certain mammalian species.

The presence of the cone visual pigments was demonstrated by monoclonal antibodies with defined specificities for the middlewave-sensitive (M) and the shortwave-sensitive (S) cone visual pigments. Retinal wholemounts and serial semithin sections were used for double labeling. The retina of the mouse, guinea pig and the rabbit is divided into a superior field that is dominated by cones containing the M visual pigment and into an inferior field with cones expressing only S visual pigment. Between the M and S fields there was a transitional zone in which cones expressed both the M and S visual pigments. The pigments showed a gradient in the intensity of their expression along the vertical meridian. In contrast to this "dual" cones, about 5-8% of all cones expressed only the S pigment (genuine S cones).

We speculate that the coexpression of the M and S pigments in the transitional cones is due to an overlap of genetic factors determining the M and S fields.

We-42 DIFFERENT RESPONSIVENESS OF SHORT AND LONG FORMS OF G_{αs} DURING (-)-ISOPROTERENOL TREATMENT OF S49 LYMPHOMA CELLS

P. Kvapil, J. Novotny, and L. A. Ransnäs

Wallenberg Laboratory for Cardiovascular Research, Gothenburg University, S-41345 Gothenburg, Sweden

Subcellular distribution and activity characteristics of G_{αs} protein (short and long forms) were studied in murine S49 lymphoma cells (wild type) during (-)-isoproterenol induced desensitization. Light (low-density microsomal fraction; LM) and plasma (PM) membranes prepared by rate-zonal centrifugation of sucrose density gradients of cell homogenates were used for these investigations. Quantitative immunoblot analysis of G_{αs} showed that short-term hormonal treatment (10 min) shifted G_{αs-short} from LM pool to PM pool and increased the total amount of membrane-bound G_{αs-long}. Long-term incubation (1 h) of cells in the presence of (-)-isoproterenol resulted in a decrease of G_{αs-short} in LM (66% of the control) and increase in PM (126% of the control) and in the increase of the total amount of membrane-bound G_{αs-long}. To examine the functional characteristics of membrane-bound G_{αs}, cyc⁻reconstitutive adenylyl cyclase activities (cyc-RACA) were measured in cyc membranes complemented with the extracts prepared from LM as well as PM. After long-term hormonal treatment the maximal cyc-RACA of the extracts prepared from LM increased to 159% or to 186% when activated by (-)-isoproterenol or AlF₄⁻, respectively. The maximal cyc-RACA of the extracts prepared from PM decreased to 65% or to 72% under the same activation conditions. These findings show that hormonal treatment raises a different responsiveness of the G_{αs} forms and changes functional characteristics of the signal transduction complex.

HYDROGEN PEROXIDE AS AN ACTIVATOR OF MACROPHAGES

We-43 I. Gamalei, K. Kirpichnikova and I. Klyubin

Institute of Cytology, Russian Academy of Sciences, 194084, St.-Petersburg, Russian Federation

A significant source of oxyradical species in intact animals and man is the respiratory burst of phagocytic cells such as granulocytes and macrophages. Being a major product of the respiratory burst of phagocytes, hydrogen peroxide (H₂O₂) is accumulated in the cell environment upon any stimulation. Our attention has been focused on the ability of small (non-cytotoxic) doses of H₂O₂ to mediate extracellular function.

The results of this study bear evidence that H₂O₂ at concentrations from 0.1 to 20 μM enhances phagocytosis and oxidative burst of murine peritoneal macrophages. The activation of these macrophage functions is paralleled by prolonged hyperpolarization and a transient increase in cytoplasmic free calcium concentration. All the effects are dose- and time-dependent. The results obtained for H₂O₂ are compared with those for a natural activator, peptide N-formyl-methionyl-leucyl-phenylalanine. The data demonstrate the ability of small doses of H₂O₂ for stimulating macrophages through the intracellular mechanisms of ion transduction.

FUNCTIONAL AND GENOMIC EXPRESSION OF THE Na,K-ATPase PUMP IN HUMAN LYMPHOCYTES ACTIVATED WITH PHA

I. Marakhova, A. Vereninov, V. Osipov

Laboratory of Cell Physiology, Institute of Cytology, Russian Acad. Sci., St. Petersburg, Russia

To elucidate the mechanism of the activation of the Na,K-ATPase pump during transit of cells from dormancy to proliferation, the relationships between changes in ouabain-sensitive K fluxes and basic proliferative indices (protein, DNA, RNA syntheses and cell growth) as well as the level of the Na,K-ATPase mRNAs have been investigated in human blood lymphocytes activated with phytohemagglutinin (PHA) for 0-72 h. A late sustained increase in ouabain-sensitive K(Rb)⁺ influx has been found which occurs after 5-8 h of PHA action and accompanies blast transformation. Unlike an early increase in Na,K-ATPase fluxes, the long-term increase is inhibited by cycloheximide and is protein synthesis-dependent. An increase in the Na,K-ATPase mRNA abundance, transient for the β mRNA and long-lasting for the α mRNA, has been observed in our studies [FEBS Lett., 1993, 316, 37-41] at the middle stage of lymphocyte activation (8-16h). The inhibition of transcription by actinomycin D or α-amanitin did not affect the cycloheximide-dependent pump flux during the first 24 h of PHA stimulation. It is concluded that all the regulatory mechanisms from rapid allosteric alterations of Na,K-ATPase to translationally and transcriptionally conditioned are involved in the ionic events during the transit of lymphocytes from dormancy to proliferation.

We-45 RECEPTOR SUPER-STRUCTURES IN THE PLASMA MEMBRANE OF LYMPHOCYTES
 S. Damjanovich, J. Matkó, L. Bene, L. Mátys, G. Vereb, G. Panyi and R. Gáspár.
 Department of Biophysics, Medical University School of Debrecen, Debrecen, Hungary

Physical association of the ICAM-1 molecule with HLA class I heavy chain, β -2 microglobulin, HLA-DR and IL-2R α was studied in the plasma membrane of HUT-102B2 and JY lymphoblastoid cell lines, using flow cytometric energy transfer measurements. Data obtained on the proximity of the above antigens, investigated two at a time by the aid of FITC and TRITC labeled mAb-s, which served as fluorescence donor-acceptor pairs, provided a nm scale distance distribution over the cell population. The co-distribution patterns made possible a two-dimensional mapping of the antigens relative to each other. Various combinations of the labeled antigens revealed a distribution pattern that was different for the T and B lymphoma line. The energy transfer difference of IL-2R α and ICAM-1 between the β -2 microglobulin and HLA class I heavy chain suggested a different angle of their localization relative to the subunits of the HLA class I molecule. They are supposedly closer to the β -2 microglobulin than the H chain, although they are farther apart from each other, than from the H chain. The JY cells may have a numerical variance between the densities of β -2 microglobulin and H chain of the HLA class I antigen. The IL-2R α was not in the proximity of HLA-DR on the surface of HUT-102B2 cells. The ICAM-1 and anti-DR L243 Ab were in energy transfer proximity on both T and B cell lines, about the same degree. Their distance was about the twice as large as the ICAM-1 and HLA class I on HUT-102B2 cells, and about same for both HLA-s on JY cells. Increased expression of ICAM-1 after INF γ increased, while TNF α decreased energy transfer efficiencies between IL-2R α and ICAM-1. Possibilities for three-dimensional cell surface patterns will also be discussed.

We-46 BRETYLIUM BLOCKS POTASSIUM CHANNELS IN HUMAN LYMPHOCYTES
 R. Gáspár, Jr., G. Panyi, Z. Krasznai, and S. Damjanovich
 Department of Biophysics, University Medical School, Debrecen, Hungary

Bretiyium tosylate (BT), a quaternary ammonium compound, is a widely used antiarrhythmic drug. Recently, it has been shown, that BT blocks the signal transduction via the T-cell receptor/CD3 complex while promotes the IL-2 dependent cell activation. It is well known that the response of human peripheral blood lymphocytes to mitogenic stimuli, where the T-cell receptor/CD3 complex is involved, can be blocked by K $^{+}$ channel antagonists in a dose dependent way, and the inhibition of mitogenesis correlates well with channel blocking potency. In accordance with this, we found that BT blocks the whole-cell outward K $^{+}$ current in resting human peripheral blood T-cells. The block of K $^{+}$ channels was dose dependent in the 0.05-5 mM extracellular concentration range, which correlates with the dose-response curve of the inhibition of mitogenesis by BT.

The kinetic analysis of the K $^{+}$ channel block showed that BT prolonged the rate of recovery from inactivation and accelerated the inactivation and deactivation rate of the K $^{+}$ current over the entire voltage range studied, however it did not influence the activation kinetics. Accumulation of the block developed gradually upon repeated opening of the channels by short depolarizing pulses suggesting the use dependence of the block.

Statistical analysis of single-channel data showed that the single-channel potassium channel conductance has not been affected by BT and no channel flickering has been introduced by the drug.

The reported effects of BT on T cell mitogenesis can partly be regarded due to its blocking effect on the type n K $^{+}$ channels of human lymphocytes.

We-47 LPAP, A NOVEL 32 kDa PHOSPHOPROTEIN WHICH INTERACTS WITH CD45 IN HUMAN LYMPHOCYTES

B. Schraven^a, D. Schoenhaut^b, E. Bruyns^a, G. Koretzky^c, S. Ratnofsky^b and S.C Meuer^a.

^aDKFZ, Appl. Immunol. Im Neuenheimer Feld 280, D-69120 Heidelberg, FRG., ^bBASF Bioresearch Corporation, 100 Research Drive, Winchester, MA 01605, USA, ^cUniversity of Iowa, College of Medicine, Iowa City, IA 52242, USA.

CD45, a leukocyte specific protein tyrosine phosphatase involved in signal transduction, associates with a 32 kDa phosphoprotein in human T-lymphocytes and T-lymphoma cell lines. The 32 kDa protein was purified and its coding cDNA cloned. Since expression of the protein was found to be restricted to B- and T-lymphocytes it was termed LPAP (lymphocyte phosphatase associated phosphoprotein). LPAP exists in two different forms in human T-lymphocytes both of which undergo alterations during T-lymphocyte activation. Analysis of LPAP expression in mutant T-cell lines suggests that the LPAP protein is only stably expressed if associated with portions of CD45 distinct from the phosphatase domains. In pervanadate treated human T-lymphocytes LPAP undergoes phosphorylation on tyrosine residues *in vivo*. Since tyrosine phosphorylation of LPAP is undetectable in T-lymphocytes expressing enzymatically active CD45, these data suggest that LPAP likely represents a novel substrate for CD45.

We-48 NEW APPROACH TO MODELING OF CELL CYCLE EVENTS OF LYMPHOID CELLS

Z.V. Grishina, M.G. Sergeeva

A.N.Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow, Russia.

We have developed the complex model of autocrine proliferation of lymphoid cells using computer system simulations of cell cycle events. The advantage of the method is a possibility to represent the components and processes of the system in biological language. The application of this method allows us to avoid problems arising when the cell proliferation is described by a system of differential equations.

We possess the unique computer program which is realized on the base of an algorithm put into practice of the flying operations. In the case of our model this program allows: (1) describe the kinetics of both the entrance of the cells in cycle and cell death by different functions dependent on both the conditions of cultivation and the cell number; (2) carry out monitoring of the cell groups across cycle in dependence on cycle phases duration; (3) consider the cell proliferation as a result of specific binding of the cells with their autocrine growth factor; (4) describe the balance of the factor in the system by common differential equation which puts into account the kinetics of synthesis and degradation of the factor and its spending for cell binding.

Computer modeling of autocrine proliferation of B-lymphoma Namalva cells was carried out using cell cycle parameters of these cells obtained from our experiments. Proliferation curves resulted from modeling were in accordance with our experimental data.

We-49

**ANTIPROLIFERATIVE EFFECT OF
SYNTHETIC PEPTIDES FRAGMENTS OF
INTERFERON α -2 ON HUMAN
LYMPHOID CELL LINES**

O.Targoni (a), A.Karulin (b), A.Danilkovich (a), K.Freze (a), A.Valujskikh (a), J.Romashkova (a), N.Makarova (c) and A.Kushch (c)

(a) Department of cellular physiology and immunology, Biological Faculty, Moscow State University, Russia; (b) Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia; (c) Institute of Virology, Russian Academy of Medical Sciences, Moscow, Russia

The influence of synthetic peptides corresponding to the 125-138, 126-138 and 127-138 amino acid sequences of interferon- α 2 molecule on growth of human T-lymphoblastoid cell lines MT-4 and CEM was investigated. Peptide's effects were measured by cytofluorometric analysis and by incorporation of radioactive thymidine into DNA. Specific binding of peptides with membrane receptors on the cells of the same lines was determined using radioligand assay. It was founded that peptides inhibited proliferation of both MT-4 and CEM cells. Studied peptides decreased absolute cell value in treated cultures and affect cell cycle distribution. Influence of peptides on cell cycle distribution was different for two studied cell lines: for MT-4 percentage of cells in G2/M was decreased on 11.4±3.2% and for CEM percentage of cell in G0/G1 was decreased on 15±4.8%. It was demonstrated that MT-4 and CEM cells expressed specific peptide receptors. It is important that level of antiproliferative activity of peptides is well correlated with their length and receptor binding properties.

We-50

**ROLE OF ARACHIDONIC ACID IN
REGULATION OF THE H⁺-CHANNEL OF
LYMPHOCYTES**

K. Káldi, K. Szászi, K. Suszták, A. Kapus and E. Ligeti

Department of Physiology, Semmelweis University of Medicine, Budapest, Hungary

Peripheral porcine lymphocytes contain an electrogenic H⁺-transporter in their plasma membrane, which can be inhibited by different cations and activated by arachidonic acid similarly to the electrogenic proton transporting pathway of neutrophil granulocytes. In granulocytes, the proton efflux can serve as charge compensation during the superoxide generation. In recent studies a close molecular relationship between the oxidase and the H⁺-channel was suggested. In our experiments we investigated whether the channel activity requires the presence of a functioning NADPH oxidase molecule. No superoxide production could be measured in our lymphocyte preparation stimulated either by PMA or arachidonic acid. The investigated cytoplasmic components of the functional oxidase could not be immunologically detected. These findings suggest that the H⁺-channel is not part of the superoxide generating enzyme complex. The H⁺-channel of lymphocytes can be activated by ATP (a potential ligand of purinergic receptors), DCCD (a known inhibitor of other H⁺ transport pathways) and by inhibition of the lipoxygenase enzyme. The possible involvement of arachidonic acid in these phenomena is discussed.

We-51

**OPOSSUM KIDNEY (OK) EPITHELIAL CELLS
CONVERT β -NAD⁺ INTO A NOVEL Ca²⁺
RELEASING FACTOR WITH CYCLIC ADP-
RIBOSE-LIKE ACTIVITY. K.W. Beers, E.N.
Chini, T.P. Dousa, Nephrology Research Unit,**

Mayo Clinic, Rochester, MN, USA 55905.

We have previously observed that increased NAD⁺ content in renal proximal tubules inhibits specifically Na⁺-Pi symport; however, the mechanism of NAD⁺ in this action upon the Na⁺-Pi symporter remains unsolved. H.C. Lee and associates (JBC 264:1608 1989) discovered that β -NAD⁺ is enzymatically converted into cyclic ADP-ribose (cADPR) a unique nucleotide which triggers release of intracellular Ca²⁺ (Ca²⁺) by a mechanism distinct from IP₃. We have investigated whether cultured OK cells, a model for proximal tubule epithelium, can convert NAD⁺ into cADPR which could then inhibit, via Ca²⁺ release, the Na⁺-Pi symporter. Incubation of OK cells with β -NAD⁺ (but not α -NAD⁺) resulted in generation of a Ca²⁺-releasing compound with basic properties similar to cADPR as determined by a specific sea urchin egg homogenate Ca²⁺-release bioassay. However, unlike cADPR synthesized by invertebrate tissues, the product of β -NAD⁺ generated by OK cells triggers release of Ca²⁺, not only from sea urchin homogenate but also triggers Ca²⁺ release from permeabilized OK cells and from microsomes prepared from rat brain and spleen. In other aspects such as homologous desensitization, response to inhibitors and potentiation by caffeine, the product of β -NAD⁺ generated by OK cells resembles activities of cADPR. Based on these data we posit that in mammalian cells β -NAD⁺ is converted to a compound closely related to cADPR which can trigger Ca²⁺ release from endocellular stores of not only invertebrate, but also in mammalian cells. Such a factor may serve as a second messenger similar to cADPR in signalling pathways of mammalian cells.

We-52

**UROKINASE RECEPTOR AND PROTEIN
KINASES CONTAINING DOMAINS ON
MONOCYTES.**

J.Bohuslav, V. Horejsi*, O.Majdic, U. Weidle**, W. Knapp, H. Stockinger. Inst. Immunology-VIRCC, Univ. Vienna, Vienna, Austria; *Inst. Mol. Gen., Prague, Czech Rep.; **Boehringer Mannheim, Penzberg, Germany.

Urokinase plasminogen activator receptor (uPA-R) with its ligand urokinase are responsible for invasive potential of cells of different origin. Here we analyzed properties of uPA-R as a probable signal transducer on monocytes. Detection of 56-60 kDa and 85 kDa phosphoproteins after in vitro kinase assay on immunoprecipitated uPA-R revealed association with protein kinases. Similar result was also obtained for uPA-R isolated on its ligand urokinase. Size fractionation of cell lysate by gel chromatography on Sepharose 4B showed association of uPA-R with kinase activity in void volume fraction exclusively, indicating very large size of these complexes. The physiological relevance of our findings was demonstrated by induction of tyrosine phosphorylation of several proteins in monocytes stimulated with urokinase. In conclusion, our results show that uPA-R is associated with protein kinase(s) in large complexes that may be crucial in signal transduction via uPA-R.

AN ALTERNATIVE WAY OF CD4/CD8 ASSOCIATION WITH PROTEIN TYROSINE KINASES

We-53 T. Cinek, P. Angelisová, J. Černý, I. Hilgert, V. Hořejší

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Praha 4, Vídeňská 1083, Czech Republic

Surface glycoproteins of T-cells CD4 and CD8 are known to associate non-covalently with the src family protein tyrosine kinase (PTK) lck via a well-defined motif in their intracellular domain. Recently we have described the existence of very large complexes resistant to the non-ionic detergent NP40, containing leucocyte membrane (glyco)lipids, GPI-anchored glycoproteins, PTKs and some of their substrates (*J. Immunol.* 149 (1992)2262). Now we report that these complexes in the HPB ALL T-cell line (CD4⁺, CD8⁺) contain both PTKs lck and fyn, a novel GPI-anchored glycoprotein CDw108 (m.w. 80 kDa), and small amounts of CD4 and CD8 but not other abundant transmembrane proteins. The PTK lck, CD4 and CD8 remain associated with the "GPI-complexes" even if the cell membranes are solubilized in the presence of the alkylating agent iodoacetamide which otherwise effectively disrupts the SH-dependent association between CD4/CD8 and lck. Therefore, we suggest that CD4 and CD8 can be associated with lck in an alternative way, independent of the cysteine-containing sequence. This could explain the recently described functionality of CD4 mutants defective in direct association with lck.

LARGE DETERGENT-RESISTANT COMPLEXES IN THE MEMBRANES OF HUMAN LEUCOCYTES POTENTIALLY INVOLVED IN SIGNALLING

We-54 V. Hořejší, P. Angelisová, J. Černý, T. Cinek, I. Hilgert

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 142 20 Praha 4, Czech Republic

Solubilization of T-, B- and myeloid cell lines membranes in solutions of mild detergents (NP40, CHAPS, Brij-58) yields several types of very large non-covalent complexes containing membrane components and intracellular protein kinases. One type of such complexes (membrane microdomains) detectable in all cell types examined contains mainly several (glyco)lipids, GPI-anchored glycoproteins, very few specific transmembrane proteins, intracellular protein tyrosine kinases (PTKs) and some of their substrates. Another large complex observed in several cell types contains CD45RA, small amounts of LFA-1 and an unidentified protein kinase. Yet another very large complex detected in T-cells contains TCR/CD3, CD2, CD4 and several other membrane molecules as well as PTKs. Finally, a complex containing CD19, CD21, HLA-DR and four molecules of the tetraspans family (CD37, CD53, CD81 and CD82) was obtained from B-cell lines. Generally, the complexes appeared to contain most components when Brij-58 was used for solubilization, while NP-40 dissociated them partially or completely. Our results indicate that leucocyte membranes may consist of microdomains of specific composition in which several receptors may partially share common intracellular signalling molecules.

ANALYSIS OF THE INTERACTIVE PROCESSES OF CD38⁺ CELLS AND HUMAN ENDOTHELIAL CELLS (HEC)

We-55 S. Deaglio^a, L. Calosso^a, G. Gabrielli^b, G. Garbarino^b, A. Funaro^a, U. Dianzani^a and F. Malavasi^a.

^aLaboratorio di Biologia Cellulare, ^bUniversità di Torino and ^cCentro CNR-CIOS, Torino, Italy and ^dUniversità di Novara, Italy.

Human CD38 is a pleiotropic molecule endowed with ADP ribosyl cyclase activity which is known to mediate cell activation in several models. Its *in vivo* function remains undetermined due to the lack of any evidence concerning its(s) ligand(s).

The starting point for this work was the recent observation of CD38 involvement in the adhesion between CD38⁺ cells and resting HEC, where CD38 acts in a selectin-like fashion. To validate this working hypothesis and in an attempt to define the basic question of the biological role of human CD38 *in vivo*, we have raised a panel of murine monoclonal antibodies (MoAbs) against HEC. An alternative approach included the generation of anti-idiotype anti-CD38 MoAbs. In both cases the recently devised intra-splenic immunization method was exploited. This provides an overall improved efficiency in immunization power.

The first approach consented the selection of a set of MoAbs which reacted with HEC and were capable of interfering with CD38-mediated adhesion. Out of these, MoAb MOON-1 was able to block CD38-mediated cell adhesion to various cell lines; furthermore MOON-1 proved to be an IgG₁, which reacted with a structure expressed on HEC, monocytes, platelets, and, to a lesser extend, T, B and myeloid cells. This finding is in line with recent evidence that the natural ligand of the ectoenzyme CD38 is located on HEC and on major cellular population of the blood. Analysis of these targets has demonstrated that CD38 activity is partially exerted through an internalization step. The normal CD38 metabolism also produces a soluble form which may find its natural ligand in an HEC surface molecule. This hypothesis has been corroborated by means of its naturally-occurring purified soluble CD38 and recombinant soluble CD38 produced in *Baculovirus*.

HUMAN CD38 RULES AN AUTOCRINE PATHWAY OF B CELL ACTIVATION AND PROLIFERATION.

We-56

A. Funaro^a, R. Volta^a, P. Cafforio^a, R. Granata^a, M. Forni^a and F. Malavasi^a.

^aUniversità di Torino, ^bDIBIT, Istituto Scientifico S.Raffaele, Milano; ^cUniversità di Bari, Ospedale Pediatrico Regina Margherita, Torino and Centro CIOS/CNR, Torino, Italy.

B cell surface molecules whose expression is increased during cell activation seem to be good candidates in order to study the processes of differentiation and activation of B cells after antigenic stimulation. The majority of the molecule relevant in B cell activation mechanisms described till now, are B cell specific.

The CD38 molecule is a non-lineage restricted 45 kDa surface glycoprotein which exhibits a structural and functional homology to *Aplysia* ADP-ribosyl cyclase. The CD38 is involved in the activation programs of human T cells; it is also expressed by B cells at high epitope density on early and late differentiation stages.

This report deals with the effects elicited on human B cells upon triggering of CD38 antigen by means of a specific agonistic antibody, i.e. IB4 MAb. The IB4 MAb used in soluble form induces small resting B lymphocytes to proliferate in the absence of other stimuli; the mitogenic effects become appreciable at 48 h, peak at day 3-4, and decrease successively. CD38 is not co-stimulatory with anti-IgM antibodies and is refractory to the addition of IL-1 α or IL-4, both cytokines not displaying additive or synergistic effects. On the contrary, CD38 shows an additive or synergistic effect with rIL-2 in a time dependent fashion. IB4 MAb induces proliferative signals also on B blasts obtained after 3 days stimulation with LPS; in this case the proliferation is improved by the presence of IL-4.

These results suggest that the CD38 molecule could represent a receptor for a yet unknown growth factor driving B cell activation and proliferation; a more appealing alternative hypothesis is that cyclic ADPR and ADPR may play a role as second messengers implementing specific genetic programs.

We-57

DOWN-REGULATION OF IL-1 SIGNAL BY
STAURSPORIN-SENSITIVE EVENTS IN EL4-6.1
CELLS. ANALYSIS AT THE LEVEL OF
IL-1-INDUCIBLE NUCLEAR FACTORS.
M. Bouaboula*, J.Dornand** and P.Casellas.*

*Sanofi Recherche, 371 rue du Pr.J. Blayac, 34184 Montpellier cedex 4,
** INSERM U65, Université Montpellier 2, 34095 Montpellier France.

EL4-6.1 cells activated by interleukin1 (IL-1) or phorbol myristate acetate (PMA) secrete interleukin2 (IL-2), IL-4 and express IL-2 receptors (IL-2R); the IL-1 response being much less potent than the response to PMA. We examined the effect of staurosporin (STAR), a protein kinase C (PKC) inhibitor on the induction of cytokine secretion and IL-2R expression. We observed that at nanomolar concentrations, STAR strongly potentiated (20 fold) the production of cytokines in IL-1-induced cells. By contrast, STAR was found to inhibit the production of IL-2 or IL-4 in PMA-stimulated cells and to negatively affect the PKC dependent expression of IL-2R, whatever the activator agent.

Quantitative polymerase chain reaction analysis indicated that the observed changes in IL-2 and IL-4 production or IL-2R expression were correlated with mRNA levels. We showed that STAR affected both the stability of the IL-2 transcript and stimulated IL-1-induced gene transcription. Run-on experiments indicated that the rate of IL2 transcription was markedly increased and sustained up to 24 h post IL-1-STAR treatment. Analysis of the transcriptional factors to IL-2 regulatory sequences such as TCEd, TCEp, APsd, AP1p, NFATd, NFATp, showed that only TCEd was sensitive to IL-1-STAR effect. Three nuclear complexes induced by IL-1 were detected in TCEd. STAR associated with IL-1 highly enhanced the quantity of these complexes whereas, associated with IL-1-PMA, it reduces their quantity. The relationship between the kinetic of TCEd-binding nuclear factors and IL-2 gene transcription implies that TCEd is very likely involved in IL-2 activation by IL-1-STAR.

These experiments suggest that, in EL4-6.1 cells, the IL-1 stimulatory pathway leading to cytokine production is down-regulated by STAR-sensitive events. These events which modulate at different levels the activation of transcriptional factors necessary for a full expression of cytokines could involve PKC(s) differing from PMA-activated PKC(s).

We-59

PRODUCTION AND CONSUMPTION OF BIOGENIC
AMINES IN SOME THYMUS CELLS AS POSSIBLE
MECHANISM OF IMMUNE MODULATION

Dina S. Gordon

Department of Histology, Chuvash State
University, Cheboksary, Russian Federation

In the cells of centres of lymphoid follicles in nodes, cells in pre-medullar zones of thymus glands, which are described before as places for dendrite cells & Kurloff cells - was seen an ability to produce histamine & monoamines. In other cases these cells had developed some histochemical reactions, that maybe a sign of belonging to APUD-system. In subcapsular zone of thymus, in coast zone of nodes & lien some similar cells were seen, but in expirments they developed abilities of amine-consumers and amine deponents. In other cases they looked as macrophages, so they cood be place for antigen presentation & processing. Both groups of described cells (productants and deponents) have good correlative communications with level of amines in themselves & in lymphocytes, studied by mathematical analysis of correlation of amine level in all cell groups. So we may think, that antigen processing & presentation really can depend from the "game" between these cells: in other words, between their giving or taking amines from each other to each other. We think so, cause we had seen strict connection and depending between stress stimulation, antigen stimulation and amine behaviour in these cells: for each variant of experiment and each type of antigen (B or T-depending) amine contents in these cells, and correlation of its level with neighbourhood was strictly definite.

We-58

TRANSMEMBRANE SIGNALLING CHANGES
WITH AGING

J. Seres^a, C. Leblanc^b, A. Penyige^c, T. Fülöp^b,

^aIst Dept. of Med., ^cInst. of Biology, Univ. Med. Sch. of Debrecen, Hungary, ^bDept. de Méd. et Centre de Rech. en Gérontol. Gériatr., CHUS et Hopital d'Youville, Sherbrooke, Qc, Canada,

We have recently demonstrated that we assist with aging to an alteration of the transmembrane signalling. We were interested at which level the alteration(s) could exist. The granulocytes and lymphocytes of healthy young (18-25yrs) and elderly (65-90yrs) subjects were studied. First, the receptor number on cell surface (IL-2, FMLP, soluble elastin) was investigated and was found not to change with aging. The GTPase activity was decreased with aging in PMNLs and lymphocytes, but to various extent. The Cholera toxin (CT) and Pertussis toxin (PT) dependent ADP ribosylation revealed various G proteins in PMNLs and lymphocytes, changing also with the aging process. The subtype characterization by anti G protein antibodies demonstrated quantitative changes (mainly Gi and Gq) with aging. The study of the PKC isoenzymes by Western blot demonstrated various patterns with aging in lymphocytes and granulocytes. The tyrosin kinase activity was decreased with aging under antiCD3 stimulation in lymphocytes. We conclude that an alteration of signal transduction mechanism at various levels can be demonstrated with aging and this might contribute to the altered immune response with aging.

We-60 EARLY MOLECULAR EVENTS IN RESPONSE
TO BETA INTERFERON TREATMENT IN
HUMAN T LYMPHOCYTES.

S. Mischia, A. Cataldi, I. Robuffo,
A. Di Baldassarre, R. Di Pietro and A. Antonucci.

Istituto di Morfologia Umana Normale
Università degli Studi di Chieti, Italy.

The early molecular mechanism actived by the treatment of human lymphocytes with human interferon beta have been studied. Results have disclosed an early increase respect to control of diacylglycerol (DG) levels as response to interferon treatment. This event has been found to derive from a rapid and sequential activation of phosphoinositide specific PLC and PLD suggesting a synergistic involvement of PIP₂ Hydrolysis and phosphatidylcholine breakdown in the early molecular signals generated by the IFN at plasma membrane level.

REDOX MECHANISMS IN LYMPHOCYTE ACTIVATION

We-61 C.M.Tzigaret, V.Cialăcu and L.M.Popescu
Department of Cell Biology and Histology,

"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

The intracellular events that link transmembrane signalling to gene expression and cell proliferation in lymphocytes are unknown. Recent evidence indicates that intracellular thiols and oxygen-derived free radicals are involved in lymphocyte proliferation.

1. Proliferation of normal human peripheral blood mononuclear cells (PBMC) was measured by flow cytometry after labeling with propidium iodide, and expressed as cell distribution in S stage of the cell cycle. Proliferation induced by concanavalin A (5 µg/ml) or phytohemagglutinin A (10 µg/ml) was inhibited by preincubation with thiol-blocking reagents N-ethylmaleimide (NEM), iodoacetamide (IA), or p-chloromercuribenzoate (pCMB), in a dose-dependent manner. The inhibition was partially reversed when cells were stimulated in the presence of thiol donors (50 µM): N-acetyl-cysteine (NAC) or 2-mercaptoethanol (2ME).
2. NEM pretreatment also inhibited PBMC proliferation induced with 10 µM ionomycin, but had no effect on mitogenic activity of 10 nM dibutyryl phorbol ester (DBP) plus ionomycin.
3. DBP alone (100 nM), but not ionomycin (10 µM) induced oxygen-derived free radicals generation in monocyte-depleted human lymphocytes labeled with dihydrorhodamine 123, as measured by flow cytometry after 30 to 120 minutes. However, the effect of DBP was potentiated by co-incubation with ionomycin.

These results suggest that intracellular thiols and oxygen-derived free radicals may be partners in a redox signalling pathway in lymphocytes.

EXPRESSION OF B1-6-BRANCHED N-LINKED OLIGOSACCHARIDES IN HUMAN ACTIVATED T4 AND T8 LYMPHOCYTES (1).

We-63 S. Lemaire, C. Derappe, M. Aubery and D. Néel
U. 180 INSERM, UFR Biomédicale des Saints Pères,
75006 PARIS

Activation of human T lymphocytes by phorbol myristate acetate (PMA) and leukoagglutinin from Phaseolus Vulgaris (L-PHA) results in important changes in N-glycosylation. The most important event is the increase in both T4 and T8 cells, especially the latter of L-PHA+ structures characterized by β1-6 branching of complex-type oligosaccharides. Moreover, the existence of a CD4-mediated increase of these β1-6-branched structures on positively selected T4 cells, as compared with the negatively selected ones, suggests that the presence of these structures, not detectable on T8 resting cells, could be related to stimulation events triggered by both selection methods. We find also that β1-6 N-acetylglucosaminyltransferase V, the enzyme implicated in the β1-6 branching of N-linked oligosaccharides has its activity enhanced in stimulated cells. This β1-6 branching on N-glycans, strongly associated with a metastatic phenotype in human and rodent tumors (2), is exhibited by numerous glycoproteins on stimulated cells, as shown by blot analysis. Therefore, it appears that, like malignant transformation in the rat 2 and SP1 tumor models, lymphocyte activation is associated with increased β1-6 GlcNAc branching of N-(this study) as well as O-linked oligosaccharides (3). It is conceivable that lymphocytes acquisition of β1-6-branched Asn-linked oligosaccharides by adhesion glycoproteins could induce, in some cases, changes in cell-extracellular matrix interactions and, consequently, in the migration of such cells into lymphoid or inflamed tissue. The changes could participate in the modulation of the inflammatory process and the immune response.

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STIMULATED MESOTHELIAL CELLS MEDIATE PERITONEAL MONOCYTE / MACROPHAGES ADHESION AND DIFFERENTIATION IN VITRO.

We-62 J. Müller^a, T. Yoshida^b

^aInstitute of Experimental Medicine, Studnickova 2, Albertov, PRAHA, Czech Republic; ^bTokyo Institute for Immunopharmacology Inc., Takada 3-41-8, Toshima-ku, TOKYO, Japan

All serose cavities including peritoneum are lined with a simple squamous mesothelium. Murine mesothelial cells have been studied in vitro in respect to believed interaction with peritoneal monocyte/macrophages during chronic inflammatory response. The mesothelial cells (MTC) expressed ICAM-1 and CD44 molecules involved in cell adhesion, both at low level. The expression of ICAM-1 can be significantly enhanced by the treatment with TNFα. When stimulated with EGF, MTC produced macrophage-colony stimulating activity (M-CSA). M-CSA was mainly due to M-CSF. EGF-stimulated MTC expressed 4,1kb M-CSF mRNA transcript.

Resting mesothelial cells were almost nonadherent for both resident and elicited peritoneal mononuclear leukocytes for several hours in vitro. TNFα treated MTC greatly enhanced their adhesive potential. EGF/TGF α stimulation of mesothelial cells resulted also in remarkable adhesion and migration of mononuclear phagocytes along mesothelial monolayer. Monocyte/macrophages proliferated extensively and differentiated on stimulated mesothelial cells. Lately, they were cytotoxic for mesothelial cells under certain conditions. Long term cultures in vitro enables us to study molecules involved in cellular interaction.

ACTIVATION OF CFTR CHLORIDE CHANNEL BY NITRIC OXIDE IN HUMAN T LYMPHOCYTE

We-64 Y.-J. Dong, A. C. Chao, K. Kouyama,
Y.-P. Hsu, R. C. Bocian, R. B. Moss,
P. Gardner
Dept. Molecular Pharmacology and Dept. Pediatrics,
Stanford University, Stanford, California,
94305, USA.

Regulation of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel in epithelial cells occurs by both cAMP- and cGMP-dependent pathway. Since CFTR is also expressed and regulated by cAMP-dependent phosphorylation in lymphocytes, we asked whether cGMP-dependent regulation exists in this cell type. By use of whole cell patch clamp recordings, we show that both endogenously produced and exogenous nitric oxide activate CFTR chloride currents in normal human cloned T cells by a cGMP-dependent mechanism. This pathway is defective in CF-derived human cloned T cells.

We-65 CYTOTOXICITY AND SPECIFIC EFFECTS OF TETRAHYDROISOQUINOLINES ON THE ENDOCRINE CELL LINE AtT20

I. Putscher, H. Haber, J. Fickel, A. Winkler, M. F. Melzig

Research Institute Of Molecular Pharmacology, 10315 Berlin, FRG

The properties of Tetrahydroisoquinolines (TIQ's) have been discussed for some years, especially in regard to Parkinson's disease and their possible role in the development of addiction. It has been reported that the dopamine derived TIQ Salsolinol (SAL) and Tetrahydro-papaveroline (THP) increase the voluntary alcohol intake of rats after i.c.v. application. However the mechanism of this effect is still unknown. Furthermore it must be clarified if the in vivo formation of the TIQ's occurs enzymatically or not. For this reason we have tested the enantiomers of SAL as well as racemic THP in the neuroendocrine cell line AtT20. The cells were treated in a dose dependent manner for 4 hours or 72 hours. The cytotoxicity of these compounds to the cell proliferation was examined by counting the viable cells after incubation for 72 hours. Different IC₅₀ values were determined for the optical antipodes R-SAL (86 μmol/l) and S-SAL (32 μmol/l) moreover lower doses of (R,S)-THP were more potent to inhibit the cell proliferation (IC₅₀=4.3 μmol/l). Investigations of the POMC gene expression showed a significant decrease of the POMC mRNA level caused by S-SAL. R-SAL treatment did not alter POMC mRNA levels. Both S- and R-SAL had no influence on the ACTH secretion (measured by ELISA), whereas an increase of ACTH release was observed after incubating the cells with THP.

We-67 ENDOCYTOSIS AND TRANSCYTOSIS FROM APICAL AND BASOLATERAL IN THYROID FOLLICLE CELLS

L. Weigl and V. Herzog

Institut für Zellbiologie der Universität Bonn, Germany

Endocytosis and transcytosis from the apical plasma membrane of thyroid follicle cells have been analysed using inside out follicles in suspension culture. Little is known about corresponding events from the basolateral plasma membrane. In this study horseradish peroxidase (HRP) was applied as a fluid phase marker to trace the transcytotic pathway from the basolateral to the apical cell surface using mini organ cultures freshly prepared from mouse thyroid glands. Because in this in vitro-system thyroid follicles maintain their normal architecture (Lab. Invest. 59, 281-291, 1988) the basolateral cell surfaces are directly accessible. Electronmicroscope analysis revealed that endosomes and the apical plasma membrane were both reached after 7' suggesting that a direct transcytotic transport pathway exists in thyroid follicle cells. Morphometric analyses showed that ~ 3% of the basolateral plasma membrane area was internalized per minute corresponding to a volume of ~ 1.0 μm³/min. 15% of the internalized HRP was detected in endocytic vesicles and endosomes. The rate of transcytosis from basolateral in resting conditions corresponded to the rate of transcytosis from apical. After stimulation with TSH, however, the rate of transcytosis from the apical cell surface was stimulated about 8-fold above resting values and exceeded that from the basolateral plasma membrane. The results show, that the translocation of plasma membrane under resting conditions from the apical and the basolateral plasma membrane is balanced wheras transcytosis from the apical cell surface predominates during TSH stimulation.

We-66 Nitric-Oxide(NO)-stimulated ADP-ribosylation of Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) in cultured cerebellar neurons
A.M. Di Stasi, C. Mallozzi, G. Macchia, M. Minetti.
Cell Biology Istituto Superiore di Sanita', Rome-Italy.

In cultured cerebellar neurons stimulation of ionotropic N-methyl-D-aspartate receptor (NMDA) induces the Ca²⁺/CaM-dependent activation of NO-synthase, increases of NO and cGMP accumulation. Moreover, the NO-generating compound sodium nitroprusside (SNP) stimulates the endogenous ADP-ribosylation activity in the rat brain by increasing the ADP-ribosylation of GAPDH, a glycolytic enzyme of 37 kDa. In the present study we used rat cerebellar neurons in order to investigate the possible target proteins involved in the NO-mediated intracellular signalling. Intact neurons at 10 days *in vitro* (DIV) were exposed to either NO-releasing agents SNP or 3-morpholinosydnonimine (SIN-1), and incubated with ³²P[NAD] in ADP-ribosylating conditions. The autoradiograms of SDS-PAGE gels showed an increase in the labelling of 37 kDa protein in the cytosol of granule neurons. We identified this protein by western blot analysis by using a polyclonal anti-GAPDH antibody. The labelling of GAPDH was dose-dependent either with SNP or SIN-1, the time course reached a plateau after 5' of exposure and a decrease at 60'. In contrast, neurons treated with NMDA (100 μM) showed the down regulation of GAPDH ADP-ribosylation, suggesting a regulatory role by neurotransmitter receptor agonist. Preliminary results indicate that, treatments of intact neurons with thiol-specific reagents can modulate the reversible thiol-disulfide exchange of GAPDH ADP-ribosylation level.

We-68 ECTO-NUCLEOTIDASES IN THE CAVEOLAE OF SMOOTH MUSCLE. A POSSIBLE ROLE OF CAVEOLAE IN NEUROTRANSMISSION

A. Kittel, E. Bacsy

Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

The caveolae are plasma membrane invaginations present in many kinds of cells. Their connection with neurotransmitters have been shown by the presence of receptor-like protein as well as of some ion-dependent ATPases, but whether the caveolae may have a role in neurotransmission it has not been elucidated yet.

We revealed localization of ecto-CA-ATPase and 5'-nucleotidase activity in the smooth muscle cells of guinea-pig vas deferens and ileum with a cerium-precipitation enzymatic method. The activity of Ca-ATPase was the strongest in the caveolae.

The simultaneous presence of 5'-nucleotidase activity in the caveolae support the hypothesis, that this Ca-ATPase has not a pump function but, together with 5'-nucleotidase, may play role in cascade transmission [Vizi et al., Neurosci. 50, 455. 1992] and the caveolae are specific structures that have a functional relationship with transverse tubules of striated muscle.

We-69 THE M- AND N-ACETYLCHOLINE RECEPTORS
ARE INVOLVED IN AMMONIA TOXICITY
Yu.Kaminsky and E.Kosenko

Institute of Theoretical and Experimental Biophysics RAS, Pushchino, Russia

Ammonia toxicity is known to be mediated in part by the NMDA-type of glutamate receptors. However NMDA receptor antagonists did not prevent the clonic convulsions in mice produced by acute ammonia intoxication. Previous experiments showed that ammonium salts are inhibitors of brain acetylcholinesterase in rats and mice, both *in vivo* and *in vitro*. As brain acetylcholinesterase is a part of the acetylcholine receptor machinery, we tested now some blockers of acetylcholine receptors in protection from acute ammonia toxicity. 100% of rats injected with the sublethal dose of ammonium acetate were shown to be convulsed and 79% die within 30 min. When rats were previously treated with atropin, the blocker of the M-acetylcholine receptor, a prevention of the convulsions was in 43% of animals and survival increased to 86%. Similar result was obtained with d-tubocurarine, the N-acetylcholine receptor antagonist. Atropin plus tubocurarine acted nearly synergically. Also, atropin plus tubocurarine prevented the convulsions and death in 27-36% of mice intoxicated with ammonium acetate. Data above clearly indicate that M- and N-types of acetylcholine receptors are involved in ammonia toxicity.

CYSTS OF THE PARS INTERMEDIA OF THE HUMAN HYPOPHYSIS. IMMUNOCYTOCHEMISTRY AND EVOLUTION ASPECTS.

We-70 D. Grandi, M.L. Arcari, G. Galanti, G.R. Trabattoni*
Department of Human Anatomy and Neurology*
University of Parma - Italy

Cysts and follicles are frequent and peculiar features within the parenchyma of the Pars Intermedia of the human hypophysis. In the present study, the cytology and immunohistochemistry of such formations was considered, to ascertain whether they originate from Rathke's pouch or from the adenohypophysis parenchyma, or from stomodaicum. We examined an autotopical series of 912 human hypophysis (M 580 W. 332). Serial sections were obtained and histological stains were accompanied with histochemical reactions. Thence, the research was supported with immunocytochemical determination for the antigens: Cytoheratin, EMA, CEA, Vimentin, Actin, Lectin, S-100 protein, GFAP, NSE, Neurofilaments, Synaptophysine, Chromogranin, POMC-derived peptide, Pars Distalis hormones. The PAS positive glycoprotein content of the cysts, did not react for the amyloid. The epithelial lining did invariably result as monostratified, with cuboid or flattened cytotypes. Their immunocytochemical expression was positivity towards the hypophysis hormones (POMC), that varied as with its degree, as well as with the association or not, of evidence for specificity. Further positivity resulted for the S-100 protein, chromogranin and cytoheratin. These immunophenotypical aspects of the epithelium allowed to rule out that the cysts are either of colloid nature, or enteric or respiratory; this was confirmed also by the general histological findings. In conclusion, we deem the typical cysts of the Intermediate Pars, directly derived from remnants of the Rathke's pouch, otherwise from cystic modifications of the parenchyma.

We-71 CYTOLOGICAL AND GENESIS OF SQUAMOUS CELLS NESTS OF THE HUMAN HYPOPHYSIS PARS-TUBERALIS.
D. Grandi, M.L. Arcari, G. Galanti, G.R. Trabattoni*
Department of Human Anatomy and Neurology*
- University of Parma - ITALY

In the Pars Tuberalis of the human hypophysis there are groups of squamous cells amongst the arrangement into cords and microfollicles of the parenchymal cells. Such groups are not observed in other hypophysis districts, and may be noted, albeit with varying frequency, in the anterior and posterior portions of the Pars Tuberalis. As with their mode of formation, they were interpreted as remnants of Rathke's pouch, otherwise as islets of parenchyma that had undergone metaplasia. These hypothesis are evaluated in the present study. Human hypophysis derived from autopsies were selected and examined. The histological and histochemical observation was completed with immunocytochemical reactions with the following antigens: Cytoheratins, EMA, Vimentin, CEA, Lectins, S-100 protein, Chromogranin, Adenohypophyseal Hormones. After weight determination, the selected glands were distributed according to the sex and into 10 year span classes. Squamous cell nests resulted to have become more and frequent with the progress of the age, after the sixth decade, and a decrease of the microfollicular parenchymal structures appeared as parallel to their frequency. As with the immunocytochemical aspects, the pronounced cytoheratin positivity seemed to point out a metaplastic process involving the parenchymal cells. This was confirmed also by the residual positivity of some cells, that was to overlap the positivity of the parenchymal cells, organized in cords or in microfollicles. Therefore, our findings do not allow to conceive the squamous cell nests as embryonic rests of Rathke's pouch.

HORMONE SECRETION OF CULTURED HUMAN PITUITARY CELLS.

We-72 E. Bácsy^a, I. Nagy^b, A. Gyévai^a, A. Kittel^a, M. Szabó^a, M. Kurcz^b

^aInst. of Experimental Medicine and ^bHeim Pál Pediatric Hospital, Budapest, Hungary

Mammotrophs and somatotrophs were originally described as two morphologically and immunocytochemically distinct cell types; a partial co-localization or co-secretion of the two hormones was later recognized in a number of species. The data on mammotrophs in the normal adult human pituitary are scarce and usually derived from para-adenomatous non-tumorous tissue. In order to define more closely the cell-biological status of the mammotrophs we developed primary cultures of dispersed human adenohypophyseal cells collected from the bodies of adult individuals within two hours after sudden death. The cells were responsive to 10⁻⁷ M somatostatin and bromocriptine but did not significantly react to 10⁻⁸ M somatotropin as judged by GH and PRL immunoassays in the medium. The cultures were fixed, embedded in Epon, then GH and PRL were localized with a double immunogold procedure.

Our results show that adult human pituitary cells obtained from cadavers of individuals without known endocrine alterations preserve their morphological differentiation, their capacity to release GH and PRL, and to respond with decreased release to somatostatin and bromocriptine, respectively. Cells storing one of the two hormones alone belong to two morphologically consistent cell types. Some cells in each of them contain the other hormone as well, in the same storage granule. We think therefore that no separate and consistent mammo-somatotroph cell population exists but the cells differentiated as somatotrophs or mammotrophs preserve the ability to secrete the other hormone as well, depending on normal or pathological physiological circumstances.

Acknowledgement. This work was supported by the Hungarian national grants OTKA 2917 and ETK T-10 T337/1990.

CONTROL OF THE RELEASE OF ACETYLCHOLINE BY PRESYNAPTIC MUSCARINIC RECEPTORS: ROLES OF Ca^{2+} AND DEPOLARIZATION

We-73

S. Tuček and V. Doležal

Institute of Physiology, Academy of Sciences of the Czech Republic,
14220 Prague, Czechia

Presynaptic muscarinic receptors on cholinergic nerve terminals can both stimulate and inhibit the release of acetylcholine from the terminals. There are principal disagreements on the identity of the receptors involved (particularly: which muscarinic receptor subtype is responsible for the inhibition of release) and on the molecular mechanism via which the receptors affect the release. It is generally accepted that changes in Ca^{2+} entry into the terminals are the key factor. In our experiments, Ca^{2+} -evoked release of acetylcholine from depolarized rat cerebrocortical prisms was inhibited by muscarinic agonist oxotremorine. The inhibitory effect of oxotremorine was strongly dependent on the degree of depolarization and much less so on the concentration of $[\text{Ca}^{2+}]_e$. The inhibition of release could not be overcome by raising $[\text{Ca}^{2+}]_e$ levels. These observations suggest that a voltage-dependent link at a post- Ca^{2+} -entry stage is involved in the control of release.

THE STATE OF IgM OLIGOMERIZATION DETERMINES THE EXTENT OF CARBOHYDRATE PROCESSING IN SECRETORY μ CHAINS.

S. Guenzi*, M.-M. Cals*, A. Sparvoli* and R. Sitia*

*DIBIT-HSR, Milano; ° Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

Five of the N-linked glycosylation sites of the secretory IgM μ chain are cotranslationally glycosylated, but differently processed during the maturation of the protein along the exocytic pathway. In particular, in murine serum IgM (polymers), the most C-terminal site (Asn 563) is unique in that it is found in the high mannose state, typical of ER proteins. As revealed by gel mobility and endo-H sensitivity assays, the Asn 563 site can undergo terminal processing when IgM are secreted as monomers following either 2-mercaptoethanol addition or Cys 575 mutagenesis. Carboxy-terminal mutants μ chains which lack the C-terminal site no longer differ in gel mobility, regardless from the oligomerization state in which they are secreted. Thus, it is the accessibility of the Asn 563 site to Golgi located enzymes and glycosyltransferases that determines the differential oligosaccharide processing in polymeric and monomeric IgM. These results imply that IgM polymerisation takes place in a pre-Golgi compartment.

CLONING AND EXPRESSION OF THE VESICULAR ACETYLCHOLINE TRANSPORTER

We-75

H.Varoqui**, M.F.Diebler*, F.M.Meunier*, J.B.Rand†, T.B.Usdin°, T.I.Bonner°, L.E.Eiden° and J.D.Erickson°.

*Département de Neurochimie, Laboratoire de Neurobiologie cellulaire, CNRS, 91190, Gif sur Yvette, France. +Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK73104, USA. °Laboratory of Cell Biology, NIMH/NIH, Bethesda, MD, 20892, USA.

A cDNA library from the highly cholinergic electric lobe of the marine ray *Torpedo* was screened with a probe from *unc-17* which encodes the putative *C. elegans* vesicular acetylcholine transporter (Alfonso *et al.*, 1993, *Science*, 261, 617). The isolated cDNA was then used to screen a rat PC12 library. The *Torpedo*, rat and UNC17 proteins have a high degree of identity with the vesicular monoamine transporters VMAT1 and VMAT2.

CV1 fibroblasts transfected with *Torpedo* or rat cDNA express high affinity binding sites for vesamicol, a drug which binds to and inhibits transport into *Torpedo* cholinergic synaptic vesicles. These cells become able to transport acetylcholine, and this transport is sensitive to vesamicol and blocked by drugs which inhibit the vacuolar ATPase.

This study clearly demonstrates that vesamicol binding and acetylcholine transport are borne by a single protein which belongs to the family of vesicular transporters using a proton antiport to sustain neurotransmitter uptake.

NON-QUANTAL ACETYLCHOLINE CAUSES HYPERPOLARIZATION OF THE MUSCLE FIBRE BY ACTIVATING ELECTROGENIC Na-K PUMP

F. Vyskočil^a, E. E. Nikolsky^b, H. Zemková^a and V. A. Voronin^b

^aInstitute of Physiology, Academy of Sciences of the Czech Republic, Praha, Czech Republic;
^bKazan Medical Institute and Kazan Institute of Biology, Kazan, Tatarstan, Russia

In a mouse diaphragm, with intact cholinesterase (ChE), the mean value of the resting membrane potential was significantly higher (84.8 ± 0.3 mV, mean \pm S.E.) at the endplate zone than in the extrajunctional area of the muscle fibres (82.5 ± 0.3 mV) at 22°C . This hyperpolarization of about 2-3 mV was abolished by $1 \mu\text{M}$ ouabain, indicating that it might be caused by an electrogenic Na-K pump. Tubocurarine (TC; $10 \mu\text{M}$) had no effect on this hyperpolarization. Short-term denervation (4 h), a slight increase of Mg in the bath from 1 to 4 mM and application of a Ca-free solution for 10 min led to the disappearance of the surplus polarization. All of these factors are known to eliminate TC-induced hyperpolarization in anti-ChE-treated muscles, which is considered to be a correlate of non-quantal acetylcholine (ACh) leakage. The surplus hyperpolarization is probably caused by a small continuous leakage of ACh from the nerve terminal, that might escape hydrolysis by acetylcholinesterase (AChE) and activate the electrogenic Na-K pump at the subsynaptic muscle membrane.

CALCIUM METABOLISM OF CHOLINERGIC NERVE TERMINALS

We-77 A. Parducz^a and Y. Dunant^b

^aInstitute of Biophysics, Biological Research Center, Szeged, Hungary; ^bDépartement of Pharmacology, Centre Médical Universitaire, 1211 Geneva 4, Switzerland

The function-dependent changes of calcium distribution were studied in cholinergic synapses following a brief tetanic stimulation. Calcium was shown to accumulate in the electric organ as a function of the number of impulses transmitted. After the end of nervous activity the surplus calcium was expelled with a half time of 5-10 min. Histochemical localization showed that the number of synaptic vesicles containing calcium deposits was increased after cessation of stimulus. The vesicular calcium accumulation is transient, it is returning to the control value during the recovery period. Rapid freezing combined with freeze fracture showed an increase in the number of pits in the presynaptic membranes starting at the end of the tetanus and culminating 1 min later.

The results support the hypothesis that vesicles play a role in the intraterminal calcium metabolism by sequestering the excess calcium which enter the nerve terminals during functioning and expel them afterwards by exocytosis.

We-79 ROLE OF DNA-PHOSPHOLIPIDS-Me²⁺ INTERACTIONS IN NUCLEAR ENVELOPE ASSEMBLY

V. Kuvichkin
Institute of Biochemistry and Physiology of Microorganisms of Russian Academy of Sciences, Pushchino, Moscow reg. Russia

It was shown, that nuclear envelope is formed from the membrane vesicles, preexisting in a cell after mitosis.

A lot of authors are consider, that only DNA-proteins and protein-proteins interactions are important in this process. Only few russian scientists proposed basic role of lipids in the nuclear envelope assembly.

Analysis of our early data of the study in vitro of complexes of DNA-phospholipids liposomes-bivalent metal cations (Me²⁺) showed their importance in nuclear envelope and nuclear pore complexes formation in vivo and in vitro.

Complexation of DNA with phospholipids of the membrane vesicles results in partial unwinding of DNA double helix. The single-stranded chains of DNA in our model of nuclear pore complexes are preferential sites for replication and transcription initiation. Thereby neighbouring genes have enhanced expression.

Pull of genes, neighbouring to DNA-membrane contacts (nuclear pore complexes) may determined cell differentiation, aging, carcinogenesis and another processes in a cell.

We-78 ORIGIN AND STRUCTURE OF NUCLEAR AND CYTOPLASMIC BODIES IN PLANT CELLS

E. Kordyum

Institute of Botany, Academy of Sciences, 252004 Kiev, Ukraine

Specific bodies appear permanently in cells of *Caragana arborescens* annual shoots during bud blooming and flowering. In formation of these bodies certain stages are established. A nuclear body appears the first as a derivative of the nucleolus. The body increases in size and becomes equal to the nucleolus, but differs from latter by ultrastructure and alkaline pH. In this time a cytoplasmic body appears being closely adjacent to an invagination of the nuclear envelope outer membrane, usually near the nucleus; the body is surrounded by numerous ribosomes. Both nuclear and cytoplasmic bodies consist of fibrils up 10 nm wide. A homogenous zone arises around the cytoplasmic body and mounts to 4.8 μm in radius. At the beginning of flowering a nuclear body disappears. Crystallization of homogenous zone content originates in the cytoplasmic body. Rays consisting of regularly situated fibrils radiate from the body in this zone and occupy the most its volume. At the end of flowering an intact cytoplasmic body is absent. Only disjoined parts of former rays are observed in the cytoplasm. In early fruiting no signs of them are revealed in cells.

On the basis of cytochemical tests and labeling with uridin-³H a protein nature is supposed for described bodies. Possible functional role of bodies and ways of nucleocytoplasmic interactions in their biogenesis are discussed.

We-80 DIFFERENTIAL DISTRIBUTION OF rRNA DURING POLLEN DEVELOPMENT

A. Olmedilla, B. Carretero, M.C. Fernández and M.I.Rodríguez-García

Dept. of Biochemistry, Molecular and Cell Biology in Plants, Estación Experimental del Zaidín (C.S.I.C.) Prof. Albareda 1, 18008 Granada, Spain

In situ hybridization is one of the most direct ways of examining the modulation of gene expression during development at the individual cell level. The male gametophyte is a relatively simple but extremely interesting object for the study of development. The aim of this study is to follow the distribution of rRNA during pollen development by *in situ* hybridization at optical level. Antisense and sense 18S rRNA biotin labelled probes were employed. The material used was anthers of *Olea europaea* and *Lycopersicum esculentum* in different development stages. Anthers were fixed in formaldehyde and embedded in Unicryl, a new resin especially recommended for *in situ* hybridization. Probes visualization were accomplished by incubation with antibiotin antibody coupled to 5 nm gold particles followed by a silver enhancement treatment. Ribosomal RNAs were detected in nucleolus and in the cytoplasmic ribosomes in all the stages studied. Accumulation of rRNA was especially abundant during vacuolated microspore and mature pollen grain. No substantial difference was observed between the two species.

We thank Dr M Delseny for the kind gift of the RNA clon. Supported by DGICYT Project n° PB92-0079-CO3-03

We-81 NUCLEAR ANTIGEN SENSITIVE TO ANTIMICRO-TUBULAR DRUGS
 N. Bello^a, E.S. Nadezhina^b, F.F. Severin^b,
N.A. Shanina^a and O.B. Solovyanova^c

^aBiology Department and ^cA.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow; Institute of Protein Research RAS, Pushchino, Moscow Region, Russia

The monoclonal antibody SN2-1F5 immunostained in cultured cells (HeLa, PKE, mouse and human embryo fibroblasts) microtubules, small spots in nuclei and some diffuse substance in the cytoplasm. It recognized in immunoblotting with HeLa proteins 170 and 50 kD polypeptides. After subcellular fractions isolation we have found that nuclear and microtubule - associated polypeptides were 170 kD. SN2-1F5 recognized 50 kD mRNA-binding protein and, probably, this protein had diffuse distribution in the cytoplasm. After either nocodazole or colcemide or colchicine (but not vinblastine or taxol) treatment the staining of both microtubules and nuclear spots disappeared, and 170 kD band was absent in immunoblotting. We suppose that the 170 kD protein(s) can participate the modification of genes expression after microtubules disruption. We thank Dr. Lev P. Ovchinnikov for p50 sample. This work was supported by Russian Fundamental Science Foundation, grant N 93-04-6538.

We-82 NUCLEO-CYTOPLASMIC LOCALIZATION OF NUCLEAR ANTIGENS DURING OOGENESIS AND EARLY DEVELOPMENT IN MOUSE

D. Vautier^a, E. Borsuk^b, D. Besombes^a, D. Chassoux^a and P. Debey^a

^aLaboratoire Associé INSERM (U-310)-INRA (Station 806), IBPC, 13 rue P. et M. Curie, 75005 Paris, FRANCE ; ^bUniversity of Warsaw, Department of Embryology, Krakowskie Przedmiescie 26/28, 00-927/1 Varsovie, POLAND

During oogenesis, proteins are synthesized at a high rate and stored for later use during the first embryonic cycles. These are characterized by large modifications of the nuclear and nucleolar structure and progressive derepression of transcriptional activities. We have analyzed during the period of oocyte and follicular growth, as well as during the first embryonic cell cycles, the modifications in nucleo-cytoplasmic localization of several nuclear antigens. These are proteins of the mRNA processing machinery. Results show that some of them are stored in the nuclei of oocytes. Not all are present in the pronuclei of zygotes, while a localization pattern similar to that of somatic cells is gained at the late 2-cell stage. The eventual relationship of these behaviors with the onset of embryonic transcription is discussed.

We-83 IN VITRO EFFECTS OF SODIUM SELENITE ON NUCLEAR 3,5,3'-TRIIODOTHYRONINE (T₃) RECEPTORS IN RAT PITUITARY GH₄C₁ CELL LINE AND LIVER
 J. Brtko^a, P. Filipčík^a, S. Hudecová^b, V. Šrbák^a, and A. Brtková^c

^aInstitute of Experimental Endocrinology, SAS, ^bDepartment of Molecular Biology, Faculty of Natural Sciences, Comenius University, ^cResearch Institute of Nutrition, Bratislava, Slovakia

3,5,3'-Triiodothyronine (T₃) receptors belong to the family of steroid/thyroid hormone nuclear receptors. One of the most important properties of the T₃ - nuclear receptor complex is its ability to stimulate or inhibit transcription of target genes.

The present study was undertaken in order to investigate the effect of the sodium selenite (Se^{IV}) on a) the formation of T₃ - nuclear receptor complex in rat liver; b) the growth of pituitary GH₄C₁ cells; c) the nuclear T₃α1 receptor (TRα1) expression; d) cytoplasmic protein phosphorylation, and e) the prolactin secretion in rat pituitary cell line. Se^{IV} at 0.1 μM (P<0.01) inhibited the T₃ specific binding to rat liver nuclear receptors as well as increased the rate of dissociation of the T₃ - nuclear receptor complex. Se^{IV} (up to 2.5 μM) has no inhibitory effect on GH₄C₁ cell proliferation as well as the prolactin secretion. On the other hand, 2.5 μM Se^{IV} decreases the TRα1 expression in GH₄C₁ cell line. At 1 μM Se^{IV}, significant changes in the electrophoretic profile as well as in the phosphorylation of cytoplasmic proteins were found.

In conclusion, Se^{IV} may reversibly affect the T₃ specific binding on the receptor molecule, moreover, it may affect specific processes at the pretranslational as well as at the posttranslational level.

We-84 FORMALDEHYDE DEHYDROGENASE: A NUCLEAR MATRIX-ASSOCIATED ENZYME

F.J. Iborra^a, M. Sancho-Tello^a, C. Guerrí^b, J. Renau-Piqueras^a

^aCtr. Invest. Hosp. "La Fe", ^bInst. Invest. Citol., Valencia (Spain).

Formaldehyde Dehydrogenase (FALDH) or class III Alcohol Dehydrogenase, an ubiquitous enzyme well preserved throughout evolution, catalyzes the oxidation of S-hydroximethyl glutathione complex, spontaneously formed in the reaction between glutathione and formaldehyde. FALDH is an ancestral system of elimination of low molecular weight substances, participating in a basic defense mechanism. We demonstrated that this enzyme is localized in the nucleus, besides cytoplasm, and is associated to active chromatin. The aim of the present work was to determine to which nuclear fraction FALDH is associated, trying to discern its function.

Subnuclear components were separated from rat liver, and we observed that FALDH activity was associated to nuclear matrix, (NM) whereas it was undetectable in other fractions (chromatin and chromatin-associated proteins). Western blot analysis showed a single band of 40 kD in SDS-PAGE, while 4 bands were detected by isoelectrofocusing, with a pH between 6.4 and 5.8, that were detectable only in NM fraction. Immunocytchemistry showed that FALDH was associated to NM and residual nucleolus fibers, while RNPs were clear. It is known that many NM-associated proteins bind DNA; although no known DNA-binding sequence has been found in FALDH. Purified FALDH was fractionated by isoelectrofocusing and analyzed by South-Western blot, and we observed that one band bound DNA, corresponding to the more prominent band observed in NM.

We-85 RAN-BINDING PROTEIN RANBP1 INHIBITS EXCHANGE OF RAN-BOUNDED GUANINE NUCLEOTIDE AND CO-ACTIVATES GTP HYDROLYSIS INDUCED BY RANGAPI.

F.R. Bischoff and H. Ponstingl, Division for Molecular Biology of Mitosis, German Cancer Research Center, D-69009 Heidelberg, Germany.

Ran (Ras-related nuclear protein) is a 25 kDa GTP binding protein with a very low intrinsic GTPase activity. It has been implicated in nuclear import of proteins with nuclear localization signals, mRNA processing and export, initiation of chromosomal DNA synthesis and onset of mitosis. Previously we had identified RCC1 and RanGAP1 as immediate antagonistic regulators of Ran activity [Bischoff et al., Nature 354, 80-82, 1991; Bischoff et al., PNAS 91, 1994, in press]. Using an overlay assay of blots with Ran-[γ^{32} P]GTP, we have isolated an additional modulator of Ran activity from HeLa cells, the 27 kDa RanBP1, and identified a 270 kDa RanBP2. An excess of added Ran-GDP did not compete for binding. RanBP1 stabilizes GTP bound to Ran, and specific GTP exchange on Ran induced by RCC1 is inhibited. RanBP1 itself does not activate Ran GTPase, however, it enhances the GTPase activity induced by RanGAP1 by an additional order of magnitude. The data indicate that there is a common or overlapping binding site for RanBP1 and RCC1 on Ran, and a different binding site for RanGAP1. To our knowledge we present the first example of a GTPase co-activator. Its properties make RanBP1 an interesting candidate for an effector of the RCC1-Ran pathway (Supported by the Deutsche Forschungsgemeinschaft).

We-87 CRYOELECTRON MICROSCOPY ANALYSIS OF THE NUCLEAR STRUCTURE OF ZEA MAYS

N. Sartori, L. Salamin Michel
Laboratoire d'analyse ultrastructurale, Université de Lausanne, Biologie, 1015 Dorigny, Switzerland.

Cryosection technique allows to study biological material very close to its native state because of the absence of chemical fixation and dehydration. Specimens are cryofixed by high pressure freezing and the state of specimen (vitrified or icy) can be checked by electron diffraction. Many investigations of chromatin organisation and nuclear structure *in situ* were performed on plants, and especially in meristematic regions where cells are in the different states of cell cycle. Most of these investigations were made on biological material prepared with conventional electron microscopy technique and, for the first time we try to understand these structures using cryoelectron microscopy. In the present work, we analyse the nuclear structure and ultrastructure of primary root meristem of Zea mays.

We investigate cells in interphase and premetaphase. Interphasic cells are characterized by a nucleus of regular shape with the two membranes parallel. In order to increase contrast, sections were slightly irradiated by the electron beam and blocks of dense chromatin appeared to be dispersed in the nucleus. Cells in premetaphase, show irregularly shaped nucleus with cytoplasmic invaginations of the external nuclear membrane with ribosomes attached to the cytoplasmic side. After irradiation, the nucleus shows a dense uniform aspect and blocks to that of dense chromatin are closely packed.

At higher magnification, dense chromatin has a fine granular aspect without any evident order and interchromatin space looks more rough. On the other hand, nucleolus is surrounded by a ring of fine grains and inside, the structure looks granular, similar to dense chromatin. During premetaphase, outer (and probably inner) nuclear membrane seems to be transformed into endoplasmic reticulum as identified by the presence of ribosomes. In the same time, blocks of dense chromatin (one block corresponds to one chromosome) mutually come closer. Neither 30 nm fiber are seen in chromatin blocks nor evident order can be determined even though some motives are found locally.

We-86 ASSEMBLY AND NUCLEO-CYTOPLASMIC TRANSPORT OF UsnRNPs
R. Lührmann, Institut für Molekularbiologie und Tumorforschung der Philipps-Universität Marburg, Emil-Mannkopff-Straße 2, D-35037 Marburg, Germany

Biogenesis of spliceosomal UsnRNPs involves migration of newly transcribed m⁷G capped UsnRNAs to the cytoplasm, assembly with the core snRNP proteins (denoted B/B', D1, D2, D3, E, F, and G), trimethylation to the m₃GpppN cap structure and finally transport back to the nucleus. The nuclear location signal (NLS) of U1 snRNP, as investigated by microinjection in *Xenopus laevis* oocytes, is bipartite, with the m₃G cap structure being one essential element. The second part of the NLS resides on the snRNP core RNP structure.

Surprisingly, the signal requirement for nuclear transport of U1snRNP was shown to differ between oocytes and somatic cells in that the m₃G-cap structure is no longer an essential signalling component. However, as shown by analysing the transport kinetics of m₃G- and AppG-capped UsnRNPs the m₃G-cap structure accelerates the transport significantly. As a prerequisite for a more detailed investigation of the mechanism of nuclear snRNP import and for the characterization of the transport factors involved, we have established an *in vitro* snRNP nuclear import system using digitonin permeabilized somatic cells supplemented with cytosolic extracts. Nuclear import of snRNPs, in permeabilized NRK cells supplemented with somatic cell cytosol, requires the same NLS structure as those identified in micro-injected mammalian cells. Interestingly, when the *in vitro* system was provided with cytosol from *Xenopus* oocytes instead of somatic cells, U1 and U2 snRNP nuclear import was m₃G-cap dependent. These results indicate that soluble cytosolic factors mediate the differential m₃G-cap dependence of U1 and U2 snRNP nuclear import in somatic cells and oocytes. We are currently investigating the contribution of individual core snRNP proteins to the nuclear transport.

As a prerequisite to investigate the assembly pathway of the core RNP domain, we have cloned the cDNAs for the human Sm proteins D2, D3, F and G. We have studied the interactions between these proteins and proteins E, F and B/B' in the absence of RNA by co-immune precipitation analysis of protein-protein hetero-oligomers. Proteins translated *in vitro* were used. Our results suggest that a protein-heterooligomer consisting of proteins E, F, G, D1, D2 binds first to the snRNA, after which addition of a B/B'-D3 complex takes place.

Finally, the structural requirements for the trimethylation of the snRNA's cap structure *in vitro* and the initial characterization of the UsnRNA-(guanosine-N2)-methyl-transferase will be reported.

EFFECTS OF ALCURONIUM ON SOLUBILIZED MUSCARINIC RECEPTORS

We-88 J. Musilová⁺ and S. Tuček
⁺Institute of Molecular Genetics, Institute of Physiology, Academy of Sciences, 142 20 Prague, Czech Republic

Alcuronium is a neuromuscular blocker which has been shown to increase the affinity of muscarinic receptors in the homogenates of rat heart, cerebellum and ileum to the muscarinic antagonist N-methylscopolamine (NMS: Tuček, Musilová et al., Molec. Pharmacol. 38: 674-680, 1990). In the present experiments, we investigated whether the positive allosteric action of alcuronium can also be observed on receptors after solubilization. Receptors from cardiac atria of adult pigs were solubilized with digitonin and deoxycholate and (³H)NMS was used as the ligand for binding experiments: receptor-(³H)NMS complexes were retained on glass fibre filters treated with polyethylenimine.

Alcuronium was found to diminish the K_d for (³H)NMS binding to solubilized receptors without altering their B_{max} value and to affect the kinetics of (³H)NMS binding in a way similar to that observed on receptors in homogenates. Under the conditions used for solubilization, muscarinic receptors did not interact with G proteins. It appears likely that the interaction between alcuronium and muscarinic receptors do not depend on the receptors association with membranes and on their interaction with G proteins.

**GA₃ AND THE CELL CYCLE IN THE SHOOT
APEX OF PISUM SATIVUM L.**

We-90

A. Daykin^a, D. Francis^a, I.M. Scott^b
and D.R. Causton^b, School of Pure
and Applied
Biology, University of Wales, Cardiff and ^b
Institute of Biological Sciences, University
College of Wales Aberystwyth. UK.

We measured the cell cycle and its component phases in the shoot meristem of *Pisum sativum* cv Meteor + GA₃ ($2.9 \times 10^{-4} M$) in 16 h light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) 8 h dark cycles at 20°C. Data were collected for the central zone (CZ), peripheral zone (PZ), pith-rib meristem (PRM), a sub-apical region below the PRM (SAR) and youngest leaf primordium (LP) by the percentage labelled mitoses method.

The cell cycle in the zones ranged between 28 and 40 h and was remarkably similar + GA₃. Generally, G₂ was the longest phase, followed by S-phase, G₁ and then M-phase. The cell cycle durations (h) compared with published cell doubling times: CZ > 70 (69), PZ 28 (30), SAR 28-36 (29), LP 28-32 (29) are consistent with a 100% growth fraction in the pea shoot apex regardless of the presence of GA₃. Other measurements indicate that the SAR is larger in the GA₃ plants. We are now examining the hypothesis that a primary effect of GA₃ is to increase the number of cells partitioned into the internode at inception rather than increasing the rate of cell division in the meristem.

CANCELLED

**THE CELL CYCLE IN A SLOW-TO-GREEN
MUTANT OF *LOLIUM TEMULENTUM* L**

We-91

L. Moses^a, D. Francis^a and H.J.
Oughamb, School of Pure and Applied
Biology,
University of Wales, Cardiff and ^b Department
of Cell Biology, Institute of Grassland and
Environmental Research, Plas Gogerddan
Aberystwyth. UK.

We have measured the cell doubling time (cdt), and the cell cycle (c) and its component phases in the central zone, peripheral zone, pith rib meristem and youngest leaf primordium in 12 day old seedlings of the slow-to-green mutant (nuclear gene mutation) of the C₃ grass, *Lolium temulentum*.

Compared with the controls, the cdt's and the cell cell cycles were longer in each zone of the mutant. In particular, the youngest primordium exhibited a cell cycle which was 1.8-fold longer in the mutant than in the control. Generally, G₁ phase was longer and G₂ phase shorter in each zone of the mutant. The growth fractions (c + cdt) indicated similar proportions of cycling cells in each zone of the controls and the mutant. These data are consistent in showing that a lengthening of the cell cycle in new primordia is at least one consequence of the nuclear mutation in the mutant.

**TEMPERATURE, CELL DIVISION AND
CELLULAR RESPONSES IN NATURAL
POPULATIONS OF *DACTYLIS GLOMERATA***

We-92

E.A. Kinsman^a, E. Yorke^a, C. Lewis^a,
M.S. Davies^a, D. Francis^a and H.J.
Oughamb, a School of Pure and
Applied Biology
University of Wales, Cardiff and ^b.
Department of Cell Biology, Institute of
Grassland and Environmental Research, PLas
Gogerddan Aberystwyth. UK.

We measured the effects of temperature on the shoot apex of a commercial cultivar, Sylvan, and 3 natural populations of *Dactylis glomerata*: 6265 ($38^{\circ}53'N$), 5401 ($44^{\circ}28'N$) and 5971 ($63^{\circ}09'N$) collected from a latitudinal transect within mainland Europe. Cell doubling times (cdts) and cell sizes were recorded in the the central and peripheral zones, pith rib meristem, and the two youngest leaf primordia, at 10, 20 or 30°C.

Generally, gradients of cdt's and cell sizes were recorded from the central zone to the peripheral zone or youngest primordia for each population at each temperature. However, cdt's and cell sizes were not always a reflection of the temperature change. For example, in 5401 (mid latitude) and 5971 (most northerly), the cdt at 10°C was faster and the cell number largest in the peripheral and pith rib meristem than in the corresponding zones at 20°C.

We-93

STRUCTURAL DOMAINS OF PLANT NUCLEAR DNA AS CONSTITUTIVE COMPONENT OF TOPOISOMERASE II/DNA COMPLEX

V. Solovyan and I. Andreyev

Institute of Molecular Biology and Genetics, Ukrainian Academy of Sciences, Kiev 252627, Ukraine.

We found that the treatment of agarose embedded plant nuclei with protein denaturants results in discrete self-fragmentation of intact nuclear DNA. The set of resultant DNA cleavage products involves several types of distinct DNA fragments sized up to 1-2 Million bp, being untyped for various eukaryotic representatives. The pattern of DNA fragmentation has been shown to be similar both in intact nuclei and in histone-depleted ones thus suggesting the observed DNA fragments represent DNA structural domains corresponding to the higher levels of chromatin folding.

Topoisomerase II-specific poisons teniposide and amsacrine have been shown to increase nuclear DNA cleavage while the conditions promoting the topoisomerase II-mediated reverse reaction lead to the rejoining of cleaved DNA domains.

The data presented suggest that nuclear DNA structural domains are involved in topoisomerase II/DNA complex the main property of which is its ability to mediate the cleavage/religation reaction.

We-94

INVESTIGATION OF COMPACT GLOBULAR PARTICLES FROM RAT LIVER NUCLEI TREATED WITH POLYANIONS

A.N.Prusov

A.N.Belozersky Institute of Physico-Chemical Biology
Moscow State University, Moscow 119899, Russia

Treatment of the isolated nuclei with polyanions (PA) (heparin and dextran sulfate) at the PA/DNA ratio of 0.4-1.5 in the 0.15-0.5 M ammonium acetate solution leads to rearrangement of the chromatin structure and formation of compact globular particles (GP) 40-70 nm in diameter bound by DNA-containing fibrils of variable thickness (2-10 nm). GP formation is accompanied by a loss of nucleosomal periodicity in DNA organization while the DNA content of nuclei remains unchanged. At the PA/DNA ratio of more than 1.5, destruction of the DNP-network and aggregation of GP in larger globular structures are observed. Isolated GP and larger globular structures in sucrose solution have density 1.30 g/cm³. They contain a complete set of four nucleosome core histones. GP can be reversibly unfolded by dialysis against low ionic strength solution to fibrils with granules 5-10 nm in diameter. It may be suppose the GP to be formed as aggregates consisting of complexes of histonecores with polyanions on the certain spots of the partly deproteinized chromatin.

We-95

THE STUDY OF HIGHER LEVEL CHROMATIN STRUCTURE USING PHOTO-ACTIVATED CROSSLINKING WITH BLUE LIGHT

I.Kireyev, A.Prusov, A.Yamshanov, V.Burakov, V.Polyakov

A.N.Belozersky Inst.of Physico-Chemical Biology, Moscow State University,
Moscow 119899, Russia

The effect of photo-activation of nucleic acids-intercalating fluorochromes on the structure of Chinese hamster and HeLa interphase and mitotic chromosomes was studied. The irradiation of permeabilized ethidium bromide-stained cells with blue light ($\lambda=460$ nm) causes irreversible stabilization of chromatin against low and high ionic strength. The effect is dose-dependent and depends on ethidium bromide concentration. The effect also depends on the initial state of chromatin compactization. Differentially decondensed irradiated chromosomes do not preserve their gross-morphology even after low ionic strength extraction but nevertheless relatively compact regions remain intact in these conditions. On the other hand, addition of antioxidants (β -mercaptoethanol, ditiotreitol) prevents but doesn't abolish the stabilization. The stabilization of irradiated chromosomes is thought to be a result of DNA-protein and/or protein-protein crosslinking. The electrophoresis of isolated irradiated nuclei has demonstrated marked decrease in extraction of histones and some nonhistone proteins while the level of extraction for other proteins remained unchanged (compared to unirradiated nuclei). So the rate of crosslinking seems to differ for different proteins and may reflect not only differences in their chemical structure but also their position relative to DNA. The resistance of chromatin to protease and DNase I treatment also increases substantially after crosslinking. High salt extraction of crosslinked and DNase I-treated chromosomes leads to its partial decompactization while non-extracted chromosomes retain their morphology even after excessive DNase treatment. We believe the method described can be used for probing higher level chromosome structures.

We-96

REACTION OF CULTIVATED CELLS UNDER INHIBITION OF TRANSLATION BY CYCLOHEXIMIDE (CHM)

Krokhina T.B., Garkavtsev I.V., Terekhov S.V.
Center of Medical Genetics, Moscow, Russia

The CHM-treatment of human diploid fibroblasts in the serum-free medium resulted in inhibition of protein and DNA syntheses during S-period, while RNA synthesis increased up to 130% (CHM - 0.1-2.0 mkg/ml), in G₀-period the incorporation of ³H-Uridine increased to 200% (CHM - 100 mkg/ml). The long-term treatment (48h) in the serum-free medium resulted in decreased uptake of ³H-Thymidine and ³H-Leucine during first 6 h of experiment, while incorporation of ³H-Uridine increased to 160%. After 16 h of CHM-treatment characters of protein, RNA and DNA syntheses came back to control level. The ultrastructure of these cells was examined under same conditions. The increasing of ³H-Uridine incorporation took place at the same time as the increasing of nucleolar activity and chromatin decondensation.

Some DNA alterations in HeLa cells were investigated. We revealed the qualitative composition of excised small polydisperse circular DNA from intact and CHM-treatment (1 and 50 mkg/ml) cells as well as compared the amount of individual classes of repeated sequences. The pool of repetitive DNA sequences in the fraction of small circular DNA increased in CHM-treated cells. The content of Alu-sequences increased by 2 times, "classical" satellite DNA - by 5-7 times, alpha-satellite DNA - by 3-5 times.

We - 97

ANTAGONISTS OF cAMP PHOSPHODIESTERASE ISOZYMES PDE-III AND PDE-IV INHIBIT PROLIFERATION OF RAT MESANGIAL CELLS (MC). JP Grande, K Matousovic, CS Chini, EN Chini, TP Dousa, Nephrology Research Unit, Mayo Clinic, Rochester, MN, USA 55905.

Recent reports indicate that in some cell types activation of cAMP-dependent protein kinase (PKA) can, via phosphorylation GTP-binding protein kinase Raf-1, inhibit cell proliferation controlled by mitogen-activated protein kinase (MAPK) signalling cascade. We determined that in rat mesangial cells cAMP is hydrolyzed by isozymes PDE-IV (~ 60%) and, to a lesser degree PDE-III (~ 40%). In our preceding study (Kidney Int. 1994, in press) we observed that PKA can be activated *in situ* by selective inhibitors of PDE isozyme(s) without stimulation of cAMP synthesis. We now explored whether specific antagonists of isozymes PDE-IV (rolipram, RP) and PDE-III (cilostamide, CS and milrinone, MIL) may in low concentrations inhibit MC proliferation in basal state and when maximally stimulated by epidermal growth factor (EGF); EGF (10 ng/ml) enhanced [³H]-Thymidine (Th) incorporation into MC 5-fold. Incubation of MC, controls or stimulated by EGF, with 3-10 μM CS caused marked inhibition (-68%) of Th incorporation, whereas 3-10 μM RP decreased the Th incorporation only by ~24%. Incubation with CS and RP combined caused almost complete arrest (~90%) of MC proliferation. Also, RP addition enhanced inhibitory effect of MIL. Incubation of MC with CS did not increase measurably cAMP levels, however, the *in situ* activity of PKA determined by -/+cAMP PKA activity ratio was increased. These results suggest that selective inhibitor of PDE-III (or PDE-III and PDE-IV) can suppress, via activation of cAMP-PKA system, proliferation of MC both in quiescent state and when stimulated by mitogen.

We - 99

POLYAMINE EXPRESSION DURING THE CELL CYCLE
J. Fredlund, M. Johansson and S. Oredsson
Department of Animal Physiology, University of Lund

Progression through the cell cycle is dependent on an adequate supply of the polyamines, putrescine, spermidine and spermine. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) are the rate controlling enzymes in polyamine biosynthesis and they are highly regulated. We have studied the expression of ODC and AdoMetDC mRNA, ODC and AdoMetDC activity and polyamine content during the cell cycle in Chinese hamster ovary cells. Using synchronously growing cells, obtained by the mitotic detachment technique and monitored by flow cytometry, we show that the putrescine, spermidine and spermine levels were approximately doubled during the cell cycle. Putrescine mainly during late S and G₂, spermidine continuously during the entire cell cycle and spermine mainly during G₁ and S phase. ODC was activated in late G₁, reached a plateau in S phase and a second burst in activity was observed during mid-late S. Maximal activity was found during the S/G₂ transition. The relative ODC mRNA level was doubled during the cell cycle, mainly during mid and late S phase. The results indicate that ODC was regulated mainly at a translational/post-translational level during G₁ to mid S phase and mainly at a transcriptional level during S/G₂ transition. AdoMetDC activity increased during G₁ and reached a first peak during G₁/S transition, increased again during mid S and reached a maximal level during S/G₂ transition and G₂ phase. The relative AdoMetDC mRNA level doubled mainly during G₁ and then remained almost constant during the rest of the cell cycle. The results indicate that AdoMetDC was mainly regulated at a transcriptional level during G₁ and mainly at a translational/post-translational level during the rest of the cell cycle.

We - 98

DNA TOPOISOMERASE I IN METASTATIC CANCER CELLS VERSUS NON-METASTATIC CANCER CELLS.

E. Asculai, E. Priel
Department of Immunology and Microbiology, Faculty of Health Science, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Most deaths related to cancer are due to metastases that are refractory to conventional therapies. The present work explores the major biochemical and biological differences between metastatic and non metastatic tumors. Many of the anticancer drugs used today act as inhibitors of DNA topoisomerase enzymes. These enzymes control and modify the biological state of DNA via the breakage and rejoining of DNA strands. In the current study, was found a higher activity of DNA topoisomerase I (topo I) in metastatic cells clones, compared to the non-metastatic clones from the same tumor. However, the metastatic and non-metastatic tumor cells contained equivalent levels of topo I protein. Moreover, the differences in the activity of the enzymes was not due to different responses to co-factors; there were also no differences between the metastatic and non-metastatic clones, in the mode that PKC activates the topo I enzyme by the same levels. We found that treatment with tyrosine phosphate inhibitors inhibits topo I activity in the non-metastatic cells. It is possible that variation in the regulation of phosphorylation/dephosphorylation process are responsible for the differences in the activity. We examined the sensitivity of the different cell lines to Camptotecin, a specific inhibitor of the topo I, since the enzyme is more active in the metastatic cells. The results shows that metastatic cells are much more sensitive to the inhibitor.

We - 100

CALCIUM BINDING TO HMG1 INDUCES DNA LOOPING BY THE HMG-BOX DOMAINS

M. Štrns, J. Reich and A. Kolíbalová

Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic

Electron microscopy has shown that native HMG1 could induce DNA looping or compaction in the presence (but not in the absence) of Ca²⁺. The effect of calcium on DNA looping was interpreted as calcium binding into the acidic C-domain of HMG1. Both individual DNA-binding HMG1-box domains A and B were found to be involved in DNA looping and compaction. Treatment of HMG1 with a thiol specific reagent N-ethylmaleimide inhibited the ability of the protein to induce DNA looping and compaction but not the electrostatic interaction with DNA. These results indicated that cysteine-sulphydryl groups of the HMG1-box domains A and B are specifically involved in DNA looping and compaction, and that in the absence of calcium the acidic C-domain down-regulates these effects by modulation the DNA-binding properties of the HMG1-box domains.

ASSOCIATION OF PROLIFERATING CELL
NUCLEAR ANTIGEN (PCNA) WITH CYCLIN
D1 AND CYCLIN A

We-101

E. Prosperi^a, A.I. Scovassi^b, L.A. Stivala^c, L.
Bianchi^c

^aCentro di Studio per l'Istochimica CNR, ^bIstituto Genetica
Biochim. Evol. CNR, ^cIstituto Patologia Gen. Universita',
Pavia, Italy

The insoluble form of the proliferating cell nuclear antigen (PCNA) bound to DNA synthesis sites is the form actually involved in S-phase DNA replication. Although interaction with cyclin D1 has been shown, the mechanism by which the transition from the soluble to the detergent-resistant form occurs, is not yet understood. In this study, the dependence of PCNA activation on kinase reaction, and the association with the S-phase related cyclin A, were investigated. Human fibroblasts blocked with hydroxyurea at the G₁/S phase boundary, were allowed to proceed to S phase in the presence of ³²P. The levels of chromatin-bound PCNA, as detected with immunocytometric analysis, were very high in S phase cells. Immunoprecipitation of PCNA from a nuclear pellet vs the cytoplasmic fraction showed that only the chromatin-bound form was highly phosphorylated. Association of PCNA with cyclin D1 and cyclin A were also found by immunoprecipitation of each protein from the same cellular lysate, and recognition by immunoblot analysis. These results suggest that binding of PCNA to DNA synthesis sites is mediated by a kinase reaction, and that PCNA is also associated with the S-phase related cyclin A.

IN SITU rRNA LOCALIZATION IN THE NUCLEAR BODIES
OF THE LARCH MICROSPORES

We-102

A.Goc^a, A.Majewska-Sawka^b, D.J. Smoliński^a
and A.Górska-Brylak^a

^a Institute of Biology, Copernicus University, Toruń, Poland;

^b Institute for Plant Breeding and Acclimatization, Bydgoszcz,
Poland

In the microspore of the larch (*Larix decidua* Mill.) there occur besides the main nucleolus numerous nuclear bodies, which nature and function are unknown. EM studies revealed that they consist of two structurally different zones. First one, dense, resembles the fibrillar component of nucleolus, the second zone shows loose structure similar to coiled bodies. Both zones of the nuclear bodies show affinity for silver ions, which is regarded as an indicator for presence of nucleolar proteins. Other cytochemical techniques indicate, that the nuclear bodies of the larch microspores contain also RNA.

To check if RNP nature of microspore nuclear bodies is related to rRNA we have used *in situ* hybridization method. Pea 18S and wheat 25S rRNA fragments were labelled with digoxigenine and immunogold labelling of anti-digoxigenine antibody were detected. Results, we have obtained, revealed that distribution of rRNA transcripts in microspore nucleus includes besides nucleolus also dense zone of the nuclear bodies. The coiled zone of these bodies has not shown any labelling.

We conclude that nuclear bodies in the larch microspore are related to nucleolus, but the nature of this relationship requires further studies.

EFFECT OF METHYL JASMONATE ON THE
REINITIATION OF CELL CYCLE DURING
SEED GERMINATION

We-103

A. Wereszczyńska, A. Grzesiuk and
M. Godlewski

Laboratory of Plant Anatomy and Cytology,
Agricultural and Pedagogic University,
Siedlce, Poland

Methyl Jasmonate (JA-Me; 0.01-0.25 mM) - a natural plant growth inhibitor - does not have any significant effect on germination of *Vicia faba* ssp. *minor* and *Pisum sativum*, slightly delays germination of *Glycine max* and markedly decreases the rate of germination and the number of germinating seeds in *Trifolium album* and *Raphanus sativus*. Inhibition of an embryo axis elongation in *Trifolium album* and *Raphanus sativus* by JA-Me was caused by (1) delayed initiation of S phase and mitosis and (2) decrease in the number of cells incorporating ³H-thymidine and in the number of mitotic cells. The intensity of ³H-thymidine incorporation was also smaller in the presence of JA-Me.

NUCLEAR DNA ENDOREPLICATION AND
CYTODIFFERENTIATION IN PLANTS.
COMPARISON OF THE EFFECT OF HYDROXYUREA ON DNA CONTENT AND CELL

We-104

^aM. Godlewski, ^aA. Bielecka and
^bA. Wereszczyńska

^aLaboratory of Plant Cytology and Cytochemistry, University of Łódź, Łódź, Poland;

^bLaboratory of Plant Anatomy and Cytology, Agricultural and Pedagogic University, Siedlce, Poland

The relationship between nuclear DNA content and cell sizes in cortex parenchyma, epidermis and hair cells of root in 6 dicotyledonous species with different 2C DNA content was determined. In *Vicia faba* ssp. *minor* and *Helianthus annuus* differentiated cells contained 2C DNA (rarely 4C DNA), in *Pisum sativum* - 8C DNA, in *Cucumis sativus* - 16C DNA and in *Raphanus sativus* and *Lepidium sativum* - 32C DNA. Higher levels of DNA content were observed in cortex parenchyma and hair cells. Hydroxyurea (HU) decreased the rate of elongation of roots and root hairs. This effect was connected with the decrease in the size of differentiated cells and with the lowered levels of nuclear DNA. After incubation in HU a greater number of cells with incomplete endocycles of DNA replication was observed.

**ULTRASTRUCTURAL DISTRIBUTION OF PCNA
DURING PLANT DEVELOPMENTAL PROCESSES**

We-105

P. González-Melendi¹, P.S. Testillano^{1,2} and M.C. Risueño¹

¹Laboratory of nuclear organization in plant development. Centro de Investigaciones Biológicas, CSIC. Madrid, Spain. ²Dep. CC. Morfológicas y Cirugía. Univ. de Alcalá. Alcalá de Henares, Spain.

The proliferating cell nuclear antigen (PCNA) is an auxiliary protein for the DNA polymerase δ, being thus a marker of DNA replication sites. Its presence has been reported to be associated with the S phase of the cell cycle in animal cells.

We have localized this antigen for the first time in plants at the ultrastructural level using a commercial monoclonal antibody on Lowicryl sections. *Allium cepa* root meristems and *Capsicum annuum* pollen grains were used. This antibody labels plant cells at the S phase of the cell cycle in proliferating tissues. It also detects periods of replication during plant developmental processes like the pollen grain formation. Gold particles revealing PCNA appear in small clusters at the periphery of the condensed chromatin masses in regions displaying decondensed chromatin fibres.

The ultrastructural localization of PCNA also enables the *in situ* study of different functional states of chromatin. This method represents an advantage over other methods that involve the incorporation of DNA markers, especially in the developmental processes, as those of pollen grain maturation, with a long interphase. Moreover, the localization of PCNA and cyclins allows the mapping of different cell cycle periods.

Work supported by Project: DGICYT PB 92-0079-C03-01

We-107 HETEROGENEITY OF MAST CELL-MACROPHAGE

**INTERACTIONS IN VARIOUS ORGANS :
DIFFERENT EFFECT OF MACROPHAGE STIMULATION
ON FUNCTIONAL STATE OF RAT GASTRIC MUCOSAL
MAST CELLS AND PERITONEAL FLUID MAST CELLS**

V.A. Botchkarev, V.S. Kupriyanov

Department of Normal Physiology, Chuvash State University Cheboksary, Russian Federation

It was investigated the functional state of rat gastric mucosal mast cells and histamine, serotonin, heparin content in peritoneal fluid mast cells after injections of prodigiosan (lipopolysaccharide, 60 mcg/kg). We determined biogenic amine content in peritoneal fluid mast cells using cytospectrofluorometric methods with o-phthalaldehyde and paraformaldehyde (histamine and serotonin, respectively), heparin content - using cytospectrofluorometric method with berberine sulfate. Prodigiosan injection causes the increase of degranulation of mucosal mast cells and decrease of its number. Also prodigiosan causes the decrease of histamine and serotonin content and increase of heparin content in peritoneal fluid mast cells without increase of their degranulation. Results indicate about heterogeneity of macrophage-mast cell interactions in various organs and tissues.

We-106 DNA SYNTHESIS IN PARTHENOGENETICALLY ACTIVATED BOVINE OOCYTES

E. Soloy, D. Viuff and T. Greve

Department of Clinical Studies, Reproduction, The Royal Veterinary and Agricultural University, Bülowsvej 13, 1870 Frederiksberg C, Denmark

Activation of nuclear DNA synthesis after fertilization is one of the important events during early embryonic development. It is difficult, however, to determine the start and duration of the DNA synthesis in zygotes as the moment of fertilization is not precisely known. Since parthenogenetically activated oocytes exhibit similar characteristics to fertilized ones and the exact moment of their activation can be controlled, we used this model to study the progression of DNA synthesis.

Bovine oocytes matured *in vitro* were activated by Ca²⁺-ionophore (10 μM, 5 min) at 30 and 36 h after the onset of maturation. Activated oocytes were either cultured for 6 h and evaluated after aceto-orcein staining or incubated with ³H-thymidine (0.37 MBq ml⁻¹, 2 h) during a period 4-16 h post-activation, spread and fixed for autoradiography.

The rates of activation (100%) and of fully formed pronucleus (98%) were similar in both 30 and 36 h oocytes. The nucleus labelling of young oocytes (30 h) examined 6, 8, 10, 12, 14 and 16 h post-activation was 15, 88, 88, 94, 53 and 12%, respectively. In aged oocytes (36 h), the corresponding nucleus labelling at the same time intervals was 58, 88, 92, 68, 34 and 3%, respectively.

In conclusion, these preliminary results indicate that the start of DNA synthesis is influenced by oocyte aging.

Supported by The Danish Research Academy

We-108 GAP JUNCTIONS, CYTOSKELETON AND HORMONAL CONTROL OF cAMP HOMEOSTASIS OF HUMAN LUTEAL-GRANULOSA CELLS

C. Fung^a, L. Cronier^b, C. Poiro^c, A. Malassine^b and D. Saltre^{cd}

^aINSERM U361, Paris, ^bPURA CNRS 290, Poitiers, ^cHisto-Embryologie-Hopital Cochin, Paris; ^dBiochimie Hormonale des Maternités, Hopital Cochin, 123 Bd Port-Royal 75014 Paris, France

The initial biochemical events associated with luteinizing hormone (LH) and follicle stimulating hormone (FSH) action are held to be result of heterotrimeric Gs-protein-sensitive adenylyl cyclase activation. With respect to granulosa cells (GC) maturation and proliferation, FSH likely in concert to growth factors is stimulatory while LH preovulatory surge acts as a "switch" preventing cell proliferation and inducing luteal differentiation. The explanation for this paradox is unclear. Human luteal-granulosa cells collected by follicle aspiration during oocyte retrieval were cultured after dispersion. Ultrastructural and indirect immunofluorescence studies with anticonnexin 43 antibodies revealed circular gap junctions in addition to gap junctional elements in the area of contact between some neighboring cells and along connecting cytoplasmic processes resting on the cell body of an other cell. Heptanol treatment-sensitive recovery of fluorescence after photobleaching was observed using FRAP method. The increase of intracellular cAMP was correlated with reversible shape changes of some cells due to disappearance of stress fibers and reorganization of microtubules while cell-cell contacts, cytoplasmic processes and gap junction-mediated dye coupling were maintained. Nocodazole treatment induced microtubule disassembly, affected cell shape, induced retraction of some long pseudopods and a different distribution of connexin 43. Thus, cAMP may not be the only second messenger able to induce dramatic cytoarchitecture changes and removal of cell-to-cell communication in the time of ovulation. In fact, luteal-granulosa cells expressed not only α subunits of heterotrimeric G proteins known to regulate adenylyl cyclases but also Gα subunits able to control PI-phospholipases C or ions channels in other cell types. The functional consequence of cell-cell communication regulation might be the control of cAMP gradients within preovulatory follicle cells. G proteins dependent-control of gap junction cAMP homeostatic buffering role is intriguing since it may account for puzzling aspect of cAMP intracellular accumulation on proliferation or differentiation of GC with regard of the cell position within follicle and cAMP-dependent protein kinases compartmentalization likely controlled by microtubules.

We-109 CELL TO CELL CONTACTS CONTROL THE CELLULAR LEVEL AND THE TRANSCRIPTIONAL ACTIVITY OF THE GLUCOCORTICOID RECEPTOR

S. Reisfeld and L. Vardimon

Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel

Contact interactions between glia and neurons are required for hormonal induction of glutamine synthetase in Müller glial cells. Glucocorticoids induce a pronounced increase in glutamine synthetase gene transcription in the intact retinal tissue but not in separated retinal cells. However, if the separated cells are reaggregated and glial cells reestablish contacts with neurons, glutamine synthetase inducibility is restored. This study examines the possible involvement of the glucocorticoid receptor (GR) in cell contact control of glutamine synthetase induction.

Using the glucocorticoid inducible reporter construct, pΔG46TCO, and control constructs that are not inducible by glucocorticoids, we demonstrated that the trans-activating capability of GR markedly declines upon cell separation. Analysis of GR protein revealed that cell separation results in a pronounced decrease in GR expression. This decrease temporally correlated with the decline in glutamine synthetase gene transcription. Overexpression of GR by transfection of a GR expression vector or activation of endogenous GR molecules by 8-bromo-cAMP enhanced the responsiveness of separated retinal cells to glucocorticoids.

These results demonstrate that expression and transcription activity of the receptor protein depend on contact interactions between retinal cells and suggest that GR is involved in cell contact control of glutamine synthetase induction.

We-110 A SCANNING AND TRANSMISSION ELECTRON MICROSCOPE (SEM AND TEM) ANALYSIS OF THE ENDOTHELIAL LINING IN THE THORACIC RAT AORTA IN EARLY STAGE OF REGENERATION - THE ROLE OF ENDOTHELIAL PROCESSES AND SUBENDOTHELIAL SUBSTANCE

M.E.Sobaniec-Lotowska, H.F.Nowak, S.Sulkowski and J.Ozięciol
Department of Pathological Anatomy, Medical Academy, Bialystok, Poland

In the work an attempt was undertaken to evaluate ultrastructural examinations (in TEM and SEM) of endothelium and subendothelial space in the thoracic aorta of Wistar rats one, two and three months after removing atherogenic diet rich in animal fat, cholesterol and methyl thiouracil. The animals were feeding with this diet for three months. No distinct signs of regression of atherosclerotic changes were observed in the internal membrane one month after removing the atherogenic diet. Two and three months after removing the applied diet the atherosclerotic changes were still observed in 1/3 of the rats. But in 2/3 of the animals regression of the atherosclerotic changes connected with reconstruction of small and shallow defects in the endothelial lining and decrease of the thrombotic changes were distinct. Regenerated cells of the endothelium ("dark endothelial cells") included a relative increase of the number of some cellular organelle (mainly microvilli, vesicles, Weibel-Palade bodies, Golgi apparatus, rough endoplasmic reticulum) and cytoplasmic processes. Numerous cytoplasmic delicate finger-like processes were protruded from the preserved endothelium towards the shallow defects and covered them as a mesh. A beginning process of the regeneration also concerned small defects in the transverse intercellular bridges. The subendothelial space below the regenerating endothelium was generally widened and it included microfibrillar substance from which the basal membrane of the endothelial cells probably arised during the process of regeneration.

We-112 ROLE OF SELF-ORGANIZATION FOR NEOPLASTIC GROWTH:

RELEVANCE FOR DIAGNOSIS AND THERAPY

Bernhard Wolf and Michael Kraus
AG Medizinische Physik und Elektronenmikroskopie,
Institut für Immunbiologie, Albert-Ludwigs-Universität Freiburg,
Stefan-Meier-Str. 8, 79104 Freiburg i. Br., FRG.

The cell is a complex system functioning outside a thermodynamic equilibrium. The prediction of cellular functions based exclusively on molecular evidence is still impossible because of the large number of interacting molecules and the non-linear interactions between cellular subsystems. However, the system cell displays a surprisingly small spectrum of macroscopically observable degrees of freedom. Their number and characteristics are different in normal and neoplastic growth. As illustrated by image analytical, microanalytical and other cell biological data we demonstrate that certain cellular observables – termed 'order parameters' – may serve as indicators for normal and neoplastic growth. The data indicate that the transition between normal and neoplastic growth can be understood as a phase transition of the mitogenic signal transduction network. In this interpretation the order parameters of normal and neoplastic cells are the result of cellular self-organization. In addition, evidence is provided that the concept of order parameters may also be fruitful for the characterization of the multistep process of cancer development. Especially, microenvironmental pH appears to play a central role for the regulation of (a) tumor cell growth and proliferation, (b) immune response, and (c) tumor cell invasion.

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CANCELLED

We-113 TRANS-MEGAKARYOCYTIC BONE-MARROW CELLS
TRANSPORT IN BRAIN HYPOXIA IN RATS

L. Dzieciol^a, W. Dębek^b, L. Chyczewski^a, J. K. Kiryczuk^b,
S. Sulkowski^a and J. Nikliński^c

^a Department of Pathological Anatomy, ^b Pediatric Surgery and ^c Thoracic Surgery, Bialystok Medical School, Poland

Megakaryocytes (MK), together with pericytes, reticular cells, macrophages, and adipocytes form the outer layer of sinusoids, contributing to the marrow-blood barrier. Transcellular passage of the hematopoietic cells from the marrow into the circulating blood is an alternative route to the migration via the intracellular gaps. However, an exact role of the emperipolesis remains unknown.

An evaluation of frequency and intensity of the megakaryocytic emperipolesis in the rats bone marrow after the unilateral carotid artery occlusion was performed. The results were compared with those obtained in the group undergoing a hemorrhage and the group not subjected to surgical procedures.

A significant increase in the frequency of emperipolesis was found in the rats bled following a carotid artery cannulation, but also in animals exposed to the carotid artery occlusion only.

The cells passing through the MK cytoplasm were examined by TEM. The cytoplasm of megakaryocytes most frequently revealed the occurrence of granulocytes, and erythroblasts. There was no fusion of the cells membranes, there was no destruction of the engulfed cells or phagosome formation in the MK. The "visiting" cells were separated from the MK cytoplasm by a narrow pericellular space, and were delivered from the intracellular canalicular system to the sinuses lumen.

The observed emperipolesis phenomenon is likely to be a result of brain hypoxia and a secondary neurogenic stimulation of the bone marrow activity, which may confirm existence of a central neurogenic mechanism regulating the blood marrow cells releasing.

We-114 CELL PROLIFERATION INDUCED BY EPIDERMAL GROWTH FACTOR IN PREFORMED MONOLAYERS OF THYROID EPITHELIAL CELLS IS INSENSITIVE TO AMILOLIDE

Lars E Ericson, Ramez Bahar, Mikael Nilsson, Dept. Anat. & Cell Biol. Univ. of Göteborg, Medicinaregatan 3, S-41390 Göteborg/Sweden.

Cytosolic alkalinization via stimulation of an amiloride-sensitive Na^+/H^+ exchanger in the plasma membrane is inferred a role as mediator of mitogenic signals. It is well-known that untransformed epithelial cells in culture cease to grow when close cell-cell contact is established (=contact inhibition). Amiloride is known to inhibit the proliferation of subconfluent thyroid epithelial cells in culture stimulated with epidermal growth factor (EGF). Here we have examined the effects of fetal calf serum (FCS), EGF and amiloride on the growth of porcine thyrocytes before (preconfluence) and after (postconfluence) the establishment of a tight cell monolayer in Transwell culture.

Preconfluence. 10% FCS was needed to grow the cell to confluence. EGF (10 ng/ml) could not replace FCS and had only little further stimulatory effect on [³H]thymidine incorporation. Amiloride (0.1 mM) fully inhibited the FCS-induced [³H]thymidine labelling and the cells remained subconfluent. **Postconfluence.** The basal [³H]thymidine incorp. was markedly reduced after a monolayer was established and 10% FCS had no further stimulatory effect. EGF increased [³H]thymidine labelling 5–10 times and the DNA content with ~50%, while the epithelial barrier (transepithelial resistance and [³H]inulin flux) remained intact. Amiloride added to either of the apical and basal media did not interfere with these EGF effects. Yet the transepithelial potential difference generated by apical Na^{2+} influx in EGF-treated cultures was abolished by amiloride.

The data indicate that EGF but not serum overcomes the contact inhibition of growth in preformed thyrocyte monolayers. The EGF-induced proliferation is, in contrast to that induced by FCS before confluence, not dependent on amiloride-sensitive Na^{2+} transport.

We-115 MORPHOLOGICAL AND BIOCHEMICAL EVIDENCE THAT CONNEXIN 32 AND CONNEXIN 43 FORM DISTINCT AND DELOCALIZED GAP JUNCTIONS IN THYROID EPITHELIAL CELLS

A. Guerrier^a, P. Fontenot^a, I. Morand^a, C. Audebert^a, D. Gros^b, B. Rousset^a and Y. Munari-Silema^a

^a INSERM U369, Fac. Méd. A. Carrel, LYON, France ; ^b UA CNRS 179, Univ. Aix-Marseille II, MARSEILLE, France

Gap junctions (Gj) are specialized regions of the plasma membrane which support the cell-to-cell exchanges of cytoplasmic molecules ($\text{Mr} < 1000$). Gj proteins (connexins or Cx) belong to a multigenic family of related but distinct proteins which have cell type-specific expression profiles. Thyrocytes express two different Cx : Cx32 and Cx43. In the present work we tried to determine a) whether these Cx are associated in the same or in different Gj plaques and b) whether their localization is related to cell polarity.

Experimental approaches included 1) double immunofluorescence (IF) labelings, on pig thyroid tissue sections, of Cx and ZO1, a tight junction-associated protein 2) western blot analysis of the Cx content of membranes separated by sucrose gradient ultracentrifugation and 3) analysis of the solubility properties of these Cx in N-Lauroylsarcosine (N-LS).

Confocal microscope examination of double IF profiles showed distinct labeling patterns for Cx32 and ZO1, and superimposable patterns for Cx43 and ZO1. Subfractionation of thyroid membranes on sucrose gradients revealed different distribution profiles for the two Cx. Cx32 was detected on membranes sedimenting between 25–35% sucrose, while Cx43 was detected with the bulk of proteins within 15–25% sucrose. N-LS treatment of thyroid membranes revealed that Cx43 Gj and Cx32 Gj presented different detergent sensitivity : Cx43 was solubilized as soon as 0.1% N-LS while Cx32 remained insoluble up to 0.4% N-LS.

Taken together these results demonstrate that 1/Cx32 Gj and Cx43 Gj are localized in different membrane regions of the polarized thyrocytes, Cx43 being localized within tight junctions and 2/the two types of Gj present differential detergent sensitivity.

We-116 CHICK LIMB DEVELOPMENT IS AFFECTED BY ALTERATION OF CHOLINERGIC RECEPTOR STATE

G. Cimoli^a, C. Angelini^b, C. Falugi^b

^aNational Institute for Cancer Research, Genova, Italy;

^bInstitute of Comparative Anatomy, University, Genova, Italy.

During limb development, cholinergic molecules were found in the interacting AER and distal mesenchyme cells, and a role was hypothesized in regulating such interaction (Drews, *Progr. Histochem. Cytochem.* 7, 1, 1975; Falugi and Rainieri, *JEM*, 86, 89, 1985).

Cholinomimetic drugs were applied during both *in ovo* early development and in limb transfilter cultures, and their effect was evaluated by morphometric, histochemical and immunohistochemical analysis. Acetylcholine receptor agonist and antagonist drugs were applied at different dilutions in chick Tyrode solution (between 10^{-5} and 10^{-9} M final concentrations) at different developmental stages: a) 24 h incubation: gastrulation and cephalic process; b) 48 h incubation: trunk and caudal neurulation. Limbs were removed at different stages during 5 more days of incubation. Transfilter cultures were performed by use of early limb ectoderm and mesenchyme, separated by type II collagenase. The drugs effect was dose-dependent, and was different according to the stage of drug application. In transfilter experiments, in the presence of inhibiting drugs, no lamellipodia were extended through the filter pores, and in *in ovo* experiments, AChR-antagonist drugs, such as *atropine*, caused limb inhibition. On the other hand, AChR-agonist drugs, such as *carbamylcholine*, caused enhanced development, but sometimes, when applied at 48 h, also anomalies. Acetylcholinesterase (AChE) activity, muscarinic receptor expression, NGF-, and protein Kinase C-like molecules distribution patterns were also altered. Inversion of the drug's effect was observed at higher dilutions or when the drugs were not injected in the yolk, but just layed on the vitelline membrane. These findings give further support to the hypothesis of a cholinergic role in the regulation of AER-mesenchyme interactions, and most probably in the regulation of the *Hox* gene expression activated by such interaction (Robert et al., *Genes Dev.*, 5, 1991).

We-117

TYROSINE PHOSPHOPROTEINS AND
NEUROTRANSMITTER RELEASE IN CORTICAL
SYNAPOTOSOMES FROM RAT BRAIN
A.Barre* E.Chieregatti* F. Benfenati^o and
F. Valtorta*

*DIBIT and University of Milan, Italy. ^oUniversity
of Rome II, Italy

Phosphorylation of proteins on tyrosine residues has generally been thought to be associated with the regulation of cell growth and differentiation. The presence of high levels of tyrosine kinase activity in adult mammalian brain, and of tyrosine phosphoproteins in synaptic vesicles, synaptic membranes, post-synaptic densities and in the synaptoplasm suggests that protein tyrosine phosphorylation may be involved in signal transduction processes, such as the regulation of neurotransmitter release. As yet, a direct relationship between tyrosine phosphorylation of synaptic proteins and synaptic activity has not been demonstrated. Here we study the release of neurotransmitter glutamate from the isolated nerve terminal (synaptosome) after activating various receptor tyrosine kinases (RTKs) with their respective ligands. Neurotransmitter glutamate release has been evoked by either by depolarizing the nerve terminals with elevated KCl, (which in this system causes a single, clamped depolarization) or by the addition of the K⁺-channel blocker, 4-aminopyridine, (which appears to cause the terminals to undergo spontaneous action potentials). We show an inhibition of Ca-dependent glutamate release upon addition of RTK ligands. The mechanism of this inhibition has been investigated by studying several parameters known to affect the release mechanism: plasma membrane potential, intracellular cytosolic calcium levels. The patterns of synaptosomal phosphotyrosine proteins and the subcellular fractionation of these proteins are also studied. In addition, the synaptosomal phosphotyrosine protein pattern reveals in the 96 and 105 kD range two proteins previously identified in synaptic vesicle preparations. These proteins are possible substrates for pp60c-src, and their purification is underway.

We-119

MoAb BD31 DEFINES A NOVEL GPI-ANCHORED
GLYCOPROTEIN CONTROLLING CELL-CELL
ADHESION IN NORMAL AND NEOPLASTIC EPI-
THELIA

Livio Trusolino ^{a,b}, Mauro Rabino ^a, Maria Prat ^a,
Ottavio Cremona ^a, Paola Savoia ^a and Pier Carlo Marchisio ^{a,b}
^a Dept. of Biomedical Sciences and Human Oncology, University of
Torino, 10126 Torino, Italy and ^b DIBIT, H San Raffaele Scientific
Institute, 20132 Milano, Italy.

MoAb BD31, raised to a line of gastric carcinoma cells, reacts with intercellular boundaries of human transformed cells originating either from carcinomas or from sarcomas growing in epithelial-like clusters as well as in primary cultures of human epithelial and endothelial cells. BD31 also reacts with intercellular rims of most normal and transformed epithelial tissues and is particularly abundant in glands and fast-growing epithelia. It is absent in nervous and muscle tissues as well as in blood and in mesenchyme-derived cells. Confocal analysis indicates that BD31 antigen is located in discrete dots at cell-cell contact boundaries and absent in basal and apical domains of cultured and *in situ* epithelial cells. MoAb BD31 precipitates a 100 kDa protein from cells labeled with ³⁵S-methionine or ³H-glucosamine as well as from ¹²⁵I surface labeled cells. This glycoprotein is resistant to trypsin in the presence of Ca²⁺ and releases an 80 kDa fragment typical of epithelial cadherins; the lack of cadherin COOH-terminal domain and protein removal by phosphoinositide-specific phospholipase indicate its membrane-anchoring by a glycosyl-phosphatidylinositol (GPI) moiety. Its extracellular domain, however, is not recognized by a MoAb to E-cadherin (HECD-1). MoAb BD31 reacts with a functionally relevant epitope and, when added to confluent cells, induces loss of cell-cell adhesion, cell scattering and appearance of migratory cell phenotypes. This GPI-anchored molecule may be a glycoprotein specifically enriched at junctions located at cellular lateral domains and with a tissue distribution similar to E-cadherin. Also in view of its functional role, it may regulate intercellular adhesion by a mechanism involving membrane-associated phospholipases.*

Supported by AIRC and by CNR target project ACRO.

We-118

INTERCELLULAR INTERACTION OF THE
NEURAL CREST DERIVATE (MELANOCYTES)
MAMMALIAN INNER EAR.

A. M. Meyer zum Gottesberge Research Laboratory,
Dept. of Otorhinolaryngology, University of Düsseldorf, Düsseldorf,
Germany

Melanocytes of the inner ear are mainly located in well vascularized areas in close proximity to the epithelial cells of the inner ear. These cells responsible for the unique endolymphatic ionic composition (150mM K⁺, 1mM Na⁺ and 2μM Ca⁺⁺) associated with the +80 mV endolymphatic potential (EP). As recently established, presence of the melanocytes is essential for inner ear function. Lack of their migration during the embryonic development causes hearing impairment and alteration or absence of the EP (Steel and Barkay 1989).

Melanocytes appeared activated under experimental hydrops conditions when endolymphatic Ca⁺⁺ concentration (10-folds increase) and EP change (Ninoyu and Meyer zum Gottesberge 1986). The melanocytes of the vestibular organ crossed the basal membrane and interacted with epithelial cells. They made simple membrane contacts associated with the formation of the vesicles and stimulation of the Golgi apparatus. These cellular alterations can be mimicked by *in vitro* incubation of the inner ear tissue in the presence of 1μM α-MSH or 1μM Ca-ionophore A 23137. These results indicate that melanocytes of the inner ear may underlie hormonal control and their intercellular interactions with the epithelial cells are related to the increase of intracellular Ca⁺⁺.

We-120

FACTORS DETERMINING THE PRESENTATION OF
PEPTIDE EPITOPE WITH MHC CLASS I

K. Eichmann, Gabi Niedermann, Maria Lucchiari,
Carola Leipner and Bernhard Maier
Max-Planck-Institut für Immunobiologie, Postfach
1169, D-7901 Freiburg, FRG

Cytotoxic T lymphocytes recognize peptides derived from proteins which are endogenously synthesized and presented with MHC class I molecules. Activation of cytotoxic activity following recognition by CTL precursors is considered to be a main mechanism in immunity against viruses and against tumor cells. Presentation of peptides with MHC class I requires the proteolytic degradation of proteins within the cytoplasm, presumably by proteasomes; this is followed by peptide transport into the endoplasmic reticulum with the help of the TAP transporters; transported peptides are then complexed with MHC class I molecules within the endoplasmic reticulum and these complexes are subsequently deposited on the outer cell membrane. Major rate limiting events in this process are the proteolytic cleavage of proteins, the peptide transport into the ER as well as the complexation with MHC molecules. We will describe studies on the role of amino acid sequences flanking potential MHC class I epitopes which suggest that these sequences may present limiting factors in the generation of the epitope by proteolytic cleavage. This rate limiting role of flanking sequences may be of importance in the determination of the degree of immunodominance of an epitope. Our studies show that a subdominant protein epitope can be converted into an immunodominant one when fitted with the flanking amino acid sequences of an immunodominant epitope. Conversely, an immunodominant epitope can be converted into a subdominant epitope when fitted with the flanking sequences of a subdominant epitope. These studies may shed a new light on the long-standing problem of epitope hierarchy in cell mediated immunity and may be useful for the design of peptide vaccines.

We-121 POLE CELLS MIGRATION THROUGH THE MIDGUT WALL OF THE *DROSOPHILA* EMBRYO
 G. Callaini, M.G. Riparbelli and R. Dallai
 Department of Evolutionary Biology,
 Via Mattioli 4, 53100 Siena, Italy.

In *Drosophila* the precursors of germ cell nuclei early segregate from the somatic nuclei and migrate to the posterior pole of the embryo, to reach a specialized cytoplasmic domain containing the determinants for the induction of the germ line. When the cellular blastoderm is completed the pole cells are passively carried to the dorsal surface of the embryo and are internalized in the posterior midgut invagination, where they form a small cluster at the bottom of the midgut sack. To reach the site of their final differentiation the pole cells must be able to migrate across the epithelial wall. Since epithelial cells are linked together by gap junctions and small apical contacts, the pole cells are expected to open these junctional complexes to travel throughout the intercellular space. The remarkable actin stain that lines the epithelial cell apex at the beginning of midgut proliferation, becomes irregular as the pole cells approach to the epithelium, suggesting a dynamic rearrangement of the apical contacts. Moreover, serial sections indicated that the pole cells push on the luminal region of the epithelial cells, modifying their surface which infolds and adapts to envelope the migrating germ cells. Prolongations of the anterior region of the pole cells reach the wide intercellular space, that characterizes the basal half of the epithelium, and the pole cell body passes into this space, moving towards the subepithelial compartment. During the migratory pathway small transitory contacts are established between pole cells and epithelial cells.

We-123 CYTOCHEMICAL INVESTIGATION OF SOME GLUCOSIDASES IN *LILUM REGALE* OVARY INFECTED WITH *BOTRYTIS CINerea*

J. D. Georgieva and R. M. Rodeva
 Institute of Genetics, Sofia 1113, Bulgaria

Localization and changes in activities of α -galactosidase and β -glucosidase in the ovary of lily (*Lilium regale* Wils) after inoculation with *Botrytis cinerea* Pers. ex Fr. were investigated. Cytochemical determination of both glucosidases was carried out by the method of simultaneous azocoupling. The parasite invaded predominantly the upper part of the ovary, penetrated through the carpel and spread to some ovules. The development of fungal mycelium provoked the appearance of necroses in the ovary and amorphous plugs in the xylem vessels of the carpel. The xylem blocking during Botrytis pathogenesis lead to poor growth conditions in the ovary. As a result delay or stopping of embryonal processes in the upper part of the ovary and failure of the fertilization in the basal ovules were observed. These disturbances in the embryonal development were accompanied by metabolic changes in ovules. The degeneration in the small unfertilized ovules gave rise to the abrupt increase of α -galactosidase activity in the embryo sac and integuments followed by the progressive decrease and loss of enzyme activity. In the ovules of the upper part of the ovary α -galactosidase and β -glucosidase activities disappeared slowly after inoculation.

Positive reaction for β -glucosidase in the cells of the carpel and integuments situated around the parasite hyphae was pointed out as a metabolic response to the fungal invasion. Since β -glucosidase is involved in breakdown of phenol glycosides and liberation of fungitoxic phenols this appearance of β -glucosidase activity could be accepted as a local defence reaction of invaded tissues. The response reaction of the generative sphere to the fungal invasion which includes not only local enzyme changes but disturbances of the metabolism in the developing ovules is more complicated than that of the vegetative tissues where local reactions generally occurred.

OOGENESIS OF STONE-FLIES, EGGSHELL
 We-122 FORMATION IN OVARY OF *PERLA*
MARGINATA (PLECOPTERA: PERLIDAE)
 (PANZER, 1779).

E. ROSCISZEWSKA

Institute of Zoology, Jagiellonian University, Kraków, Poland

The eggshell (egg capsule) of stone flies is specialized to aquatic habitat. On the posterior pole the egg capsule is equipped with an attachment structure. In the region where the attachment structure is anchored, the chorion is modified into collar. All elements of egg capsule: vitelline envelope, chorion, attachment structure and extrachorionic layer, are secreted by follicular cells. The morphological changes that these cells undergo during the secretion process, were studied. In particular the diversification of the cells into subpopulations responsible for secretion of different elements of the egg capsule is discussed.

We-124 IMMUNOREACTIVE LOCALIZATION OF THE AMIDATING ENZYME (PAM) IN DEVELOPING AND ADULT MOUSE LUNG

L. Guembe and A. C. Villaro

Departamento de Histología y Anatomía Patológica, Facultades de Medicina y Ciencias, Universidad de Navarra, 31080 Pamplona, Spain

Peptidyl-glycine alpha-amidating monooxygenase (PAM) is a single bifunctional enzyme which catalyzes the alpha-amidation of many regulatory peptides. We have studied the distribution of this enzyme in fetal, neonatal and adult mouse lung by the indirect immunocytochemical method avidin-biotin complex (ABC) technique applied to paraffin sections.

PAM immunoreactivity was localized in bronchial and bronchiolar epithelium. We have found intense immunoreactivity from gestational day 18 to adulthood. On gestational day 16, a slight immunostaining can be observed in scattered cells. The immunostaining extends throughout the cytoplasm, but in some cells is more intense in the apical region. The specificity of the stain was tested by negative and absorption controls.

In conclusion, PAM immunoreactivity is present throughout the mouse airways epithelium. The presence of PAM positivity in most of the epithelial cells may indicate that this enzyme could be involved in the chemical processing of certain, yet unknown, factors produced by the epithelial cells themselves. Considering that a more intense immunostaining is sometimes observed in the apical region, it is also possible that this enzyme is segregated into the lumen.

This study was supported by the "Comisión Interministerial de Ciencia y Tecnología" (CICYT) (Project n° PB 88-0553) and by a grant from the "Gobierno de Navarra" (L. Guembe).

We-125 IMMUNOCYTOCHEMICAL LOCALIZATION OF NITRIC OXIDE-SYNTHASE IN MALPIGHIAN TUBULES AND GUT OF TWO INSECT SPECIES: *Drosophila melanogaster* (Diptera) AND *Formica polyctena* (Hymenoptera)

L. Montuenga¹, M. Garayo¹, M. García¹, A. C. Villaro¹ and V. Riveros-Moreno².

¹Departamento de Histología y Anatomía Patológica, Facultades de Medicina y Ciencias, Universidad de Navarra, 31080 Pamplona, Spain.

²Department of Pharmacology, Wellcome Foundation Ltd., Beckenham, Kent, UK.

Recent evidence has been reported for the presence of the elements of the nitric oxide (NO) signalling pathway, and for their role in controlling fluid secretion in *Drosophila melanogaster* Malpighian tubules. In this study, the presence of NO-synthase in *Drosophila melanogaster* and *Formica polyctena* Malpighian tubules and alimentary canal has been immunocytochemically investigated, using several antibodies against constitutive NO-synthase.

Immunoreactivity has been found along the whole length of *Formica polyctena* Malpighian tubules, except for the proximal segment of the tubules draining into the ampulla. In *Drosophila melanogaster*, immunolabelling showed segmental differences along the tubules. Muscle visceral cells surrounding the midgut and hindgut were also immunoreactive for NO-synthase.

Our results strongly favour the presence of NO-synthase in Malpighian tubule epithelial cells and visceral muscle cells of insects. This would imply that in insects NO is generated by Malpighian tubules and muscle cells themselves, and does not diffuse from a nearby tissue. Besides, the presence of NO-synthase in insects also supports that the NO signalling pathway is highly conserved through evolution.

This study was supported by a grant from the EC (SCI-CT90-0480), and from the Ministerio de Educación y Ciencia (DGICYT, CE 91-0002).

We-127 INFLUENCE OF THE COCULTURE WITH BOVINE OVIDUCTAL CELLS (BOEC) AND OF THE HEPARIN TREATMENT ON THE BOVINE FROZEN-THAWED SPERM (BS) ACROSOME LOSS (%AR)

A. Kochetkov, A. Makarevich, J. Pivko, J. Bulla
Int. Biotech. Lab., Inst. Animal Production;
Hlohovska 2, 94992 Nitra, Slovak Republic

Influences of the incubations with long-term bovine oviduct epithelium cells at 8th passage (BOEC), 3-day-conditioned medium (BOEC-CM), spTALP+ 10 or 20 µg/ml heparin (HEP1 or HEP2) on the %AR (live BS only) were studied. As controls the incubations with spTALP medium (TALP), alpha-MEM culture medium (MEM), and murine embryonal fibroblast monolayer (EFM) were used. The smears had been prepared during 7-h incubation and were triple-stained. The primary data were compared by Mann-Whitney rank test.

The co-culture of sperms with BOEC and BOEC-CM ($p<0.05$) as well as the incubation with HEP1 and HEP2 ($p<0.01$) promoted increasing of %AR in comparing with all controls. We not found a significant differences between influences of BOEC and BOEC-CM, between HEP1 and HEP2 and between all controls (TALP, MEM and EFM) on the %AR. The influence of HEP2 incubation on %AR is more strong than those with BOEC-CM ($p<0.01$). The number of live BS in EFM-coculture was higher than in other samples.

We-126 NITRIC OXIDE-SYNTHASE IMMUNOREACTIVITY IN THE RESPIRATORY SYSTEM OF *Rana temporaria* (AMPHIBIA) AND *Podarcis hispanica* (REPTILIA)

C. Beorlegui¹, M. E. Bodegas¹, A. C. Villaro¹, P. Sesma¹ and V. Riveros-Moreno².

¹Departamento de Histología y Anatomía Patológica, Facultades de Medicina y Ciencias, Universidad de Navarra, 31080 Pamplona, Spain

²Department of Pharmacology, Wellcome Foundation Ltd., Beckenham, Kent, UK.

Nitric oxide (NO) has been shown to be an endogenous molecule with a role in transcellular communication, being considered as a novel neural messenger. A significant morphological evidence for the presence of NO is the demonstration of NO-synthase immunoreactivity. We have studied the distribution of this enzyme in the lung of *Rana temporaria* (Amphibia) and *Podarcis hispanica* (Reptilia) by a immunocytochemical method applied to paraffin sections.

A moderate number of nerve bundles and neuronal bodies immunoreactive to NO-synthase were detected in the pulmonary wall of *Rana temporaria*. The intensity of immunostaining varied somewhat between positive neurons. In *Podarcis hispanica* some nerve bundles occur along the trachea and in the pulmonary wall. In addition, some fibers around the neuronal bodies were immunoreactive to NO-synthase. These are the first descriptions of immunoreactivity to NO-synthase in the lung of non-mammalian vertebrates.

The presence of NO-synthase in nervous elements of the respiratory system supports the hypothesis of NO being the nonadrenergic and noncholinergic (NANC) relaxatory neurotransmitter of this system.

This study was supported by the "Comisión Interministerial de Ciencia y Tecnología" (CICYT) (Project nº PB 88-0553), and by a grant from the "Gobierno de Navarra" (M. E. Bodegas).

We-128 ION-OSMOTIC HOMEOSTASIS IN THE MIXED CELL CULTURES: THE ROLE OF GAP-JUNCTIONS

L.Ju.Boitzova*, T.V.Potapova*, K.B.Aslanidi*
"The A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University,
119899 Moscow"; Laboratory for Cellular Biophysics, Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, 142292 Moscow Region, Russia.

The mathematical model analysis developed suggests that in the multicellular system with intercellular permeable junctions (PJ) consisting of cells with (a) active and (b) completely inactive ion pumps, the ion gradients and membrane potentials can be maintained not only in (a) but also in (b) cells. Comparison between model predictions and experimental results was carried out for different mixed cultures of mammalian cells. In mixed cell cultures of human embryonic fibroblasts with one of the rodent cell lines - mouse embryonic fibroblasts, BHK-21 or rat glialia C6-membrane potentials, input resistances, intracellular ion contents and intercellular dye transfer were recorded. Low ouabain concentration completely abolished membrane potentials as well as changed intracellular Na and K of the human cells in pure culture. The same treatment did not change all these parameters of rodent cells such as BHK-21, mouse fibroblasts and C-6 in pure or mixed cultures, as well as of the human cells in mixed cultures with BHK-21 and mouse fibroblasts. But the such effect was very small in the human with C-6 mixed cultures. The best dye transfer was revealed between human and BHK or mouse fibroblasts but nearly was absent with C-6. It means that electrochemical potential gradients of ions across cytoplasmic membranes in human cells have been supported by Na-K-ATPas of rodent cells.

It is concluded that effective ion transport via PJ promotes energetic cooperation between cells.

We-129 MODULATION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION BY A TYROSINE PHOSPHATASE INHIBITOR

T. Husøy, S-O. Mikalsen and T. Sanner
Laboratory for Environmental and Occupational Cancer,
Institute for Cancer Research, The Norwegian Radium
Hospital, N-0310 Oslo, Norway.

Cycles of phosphorylation and dephosphorylation are of importance in the regulation of many cellular processes. Both the serine/threonine kinase protein kinase C (PKC) and the tyrosine kinase Src have previously been found to phosphorylate the gap junction protein, connexin43, and to reduce gap junctional intercellular communication (GJIC). We have found that the tyrosine phosphatase inhibitor pervanadate strongly decrease GJIC. The reduction in GJIC was reversible, with a recovery of GJIC after 2-3 hours. Pervanadate also increased the phosphorylation of connexin43, as seen by the increased intensity of the highest MW bands and the complete disappearance of the lowest MW band as shown by Western blotting. The normal band pattern was mainly restored after 3-4 hours. Since pervanadate is a tyrosine phosphatase inhibitor, the effect of pervanadate could be a result of increased tyrosine phosphorylation. Addition of a tyrosine specific phosphatase after pervanadate exposure and the use of a phospho-tyrosine antibody clearly indicated that pervanadate induced phosphorylation on tyrosine. Other phosphatase inhibitors like sodium molybdate, vanadyl aluminium fluoride, sodium orthovanadate, vanadyl sulphate, okadaic acid and calyculin A had no effect on GJIC. Supported by the Norwegian Cancer Society, the Research Council of Norway and grant from EC contract no. CT91-0146 (DTEE).

We-130 ACANTHOLYSIS INDUCED IN HUMAN KERATINOCTYES BY SPECIFIC INHIBITORS OF SERINE-THREONINE PROTEIN PHOSPHATASE

M. Serres, M. Haftek, M.J. Staquet and D. Schmitt
INSERM U 346, Pav R, Hopital E. Herriot, LYON,
FRANCE

Transduction pathway characterized by signal transmission from the membrane to the nucleus of the cell, plays an essential role in regulating cellular mechanisms like proliferation, differentiation and oncogenesis. The state of phosphorylation of a protein is determined by the relative activities of protein kinases and protein phosphatases. If the role of protein kinases in cell regulation has been well documented, few reports concerned the protein phosphatases. In this study we report the effect of protein phosphatases in the control of cell-cell interactions and spreading. This study was carried out on normal human keratinocytes and on a spontaneously immortalized human keratinocyte cell line HaCaT which shows highly preserved phenotypic characteristics of normal keratinocytes. Preliminary results demonstrated a high change in cell morphology (acantholysis) induced by pefabloc, okadaic acid and calyculin A selective inhibitors for Ser-Thr phosphatases, whereas orthovanadate, a potent inhibitor for Tyr-phosphatases was without effect. Other inhibitors of phosphatases such as NaF or methylamine had no effect. Our results show that the effect of the inhibitors is time and dose dependent with a possible reversible effect. The change in morphology was not prevented by TPA treatment. Extracellular Ca^{2+} cations modified only the time-dose response suggesting a primary effect of Ser-Thr phosphatase inhibitors on intracellular junctions. Results from FACS analysis show that integrins were not the main target as $\beta 1$ and $\beta 4$ integrins were only slightly decreased by inhibitor treatment whereas E cadherin expression was significantly decreased. Studies in laser confocal scanning microscopy show that the cytoskeleton was highly modified. Electron microscopy results demonstrated that the structure of mitochondria was not affected. The specific action of Ser-Thr phosphatase inhibitors demonstrates the essential role of these proteins in the regulation of cell-cell contacts and in the reorganization of the suprastructure of the cytoskeleton and intercellular junctions. The mechanism of regulation of these proteins may be directly implicated in dermatological pathologies such as autoimmune pemphigus and Hailey-Hailey's disease characterized by acantholysis and a particular fragility of epidermis.

We-131 Inhibitory action of suramin in embryonic cell interactions

Cardellini P. and Barison D.
Department of Biology - University
of Padova - Italy

Cleaving *Xenopus* eggs treated with suramin produce embryos with normal cleavage rate, but differentiation of the mesodermal tissue is suppressed.

The mesoderm normally organizes the development of the embryo. However, if suramin is used, gastrulation and other morphogenetic events do not take place (Cardellini et al. Mech. Dev. 45, 73-87; 1994).

This type of result has now also been confirmed using *Brachydanio rerio* (zebrafish) embryo: in this case, the lack of yolk sac formation usually results in early disruptions of yolk envelop and embryo degeneration. Only a few embryos survive, and show the absence of mesoderm differentiation.

Treatment is more efficient during early cleavage stages, when mesodermal induction occurs.

Suramin can be used to create cell isolation, probably by masking the receptors and/or the growth factors acting in embryonic inductions (FGF and TGF).

Future study will aim at investigating the mechanism of action of suramin, although it is believed that the capacity of this substance for protein binding is very aspecific and generic, and that it can be used to interfere with several situations encountered in intercellular communication.

We-132 PRESENCE OF ATP-CITRATE LYASE IN THE FRACTION OF ZAJDELA HEPATOMA ENRICHED WITH PROTEINS OF THE PEPTIDE TRANSLATION APPARATUS

Z. Tuháčková^a, J. Křivánek^b

^aInstitute of Molecular Genetics and ^bPhysiological Institute, Acad. Sci. of the Czech Republic, Prague

Basic amino acid histidine was found to be phosphorylated in the molecule of a major auto-phosphorylated protein present in the fraction of Zajdela rat hepatoma retained on heparin-Sepharose. Highly purified phosphoprotein revealed activity of ATP-citrate lyase that was not affected by several agents inhibiting the acid-labile autophosphorylation of the protein. The unstable molecule of the purified protein is decomposed into four identical subunits, after which the enzyme loses the ability to catalyze the formation of acetyl-CoA but not the ability to be autophosphorylated. The identification of ATP-citrate lyase, the key enzyme of the lipid biosynthesis, as one of the proteins of the fraction of Zajdela ascites rat hepatoma enriched with protein factors required for the translation of mRNA, might indicate that there is a close relationship between the supramolecular organized translation system and the lipid biosynthesis pathways in mammalian cells.

We-133 WATER-SOLUBLE DERIVATIVES OF HEPARIN FOR BINDING STUDIES OF PROTEINS FROM BOAR SEMINAL PLASMA

M. Tichá^a, M. Kraus^a, B. Železná^b, V. Jonášová^b

^aDepartment of Biochemistry, Charles University, Praha 2, Czech Republic

^bInstitute of Molecular Genetics, Academy of Sciences of the Czech Republic, Praha 6, Czech Republic

Water soluble poly(acrylamide-allyl amine) derivatives containing covalently bound amino groups were prepared by copolymerization of acrylamide and allyl amine in the absence of cross-linking agent. Heparin was coupled with its carboxyl groups to the amino groups of the prepared poly(acrylamide-allyl amine) derivative using the carbodiimide reaction. The prepared derivative of heparin was labeled either using periodate oxidized horseradish peroxidase or by biotinylation.

The prepared labeled glycoconjugates of both types containing coupled heparin were applied in the studies of binding properties of proteins from boar seminal plasma. The following techniques were used:

- a) dotting assays using nitrocellulose membranes,
- b) the detection of heparin-binding proteins on nitrocellulose membranes after isoelectric focusing and electroblotting,
- c) the detection of heparin-binding proteins immobilized to plastic microtiter wells in the enzyme-linked-binding assay (ELBA).

We-134 Overexpression of N-CAM₁₈₀ in human neuroblastoma cells induced to neuronal differentiation by retinoic acid

M.Cervello^a, L.D'Amelio^a, G.Rougon^b and V.Matranga^a

^aIstituto di Biologia dello Sviluppo, Consiglio Nazionale delle Ricerche, Palermo, Italy; ^bLaboratoire de Genetique et Physiologie du Developpement, Parc Scientifique de Luminy, Marseille, France

The role of N-CAM during nervous system development is well known. Three different isoforms have been described, with molecular masses of 180, 140 and 120 kDa, whose differential expression seems to be related to neuronal differentiation. We took advantage from the use of the human neuroblastoma cell line LAN-5, that can be differentiated *in vitro* by retinoic acid, for studying the expression of N-CAM isoforms at the protein and mRNA levels. We used anti-N-CAM polyclonal antibodies recognising all the isoforms in Western blot experiments and found that growing cells express low levels of 180 kDa N-CAM. The levels of the corresponding mRNA, determined by Northern blot using a 600bp cDNA probe corresponding to exons 10-14 of the mouse N-CAM gene, were hardly detectable. When we measured the expression of the 180 kDa N-CAM isoform in LAN-5 cells differentiated with retinoic acid for 14 days, we found a 5-fold increase in the protein content and 20-fold increase in the 7.2 kb corresponding mRNA level. On the contrary, growing cells expressed high levels of the 140 kDa N-CAM isoform, whereas there was only a modest increase in the expression of the protein and the mRNA upon differentiation, that could be calculated as a 1.4-fold and 2.5-fold increases respectively. We were not able to detect the 120 kDa isoform either in growing or differentiated cells. In conclusion our data show an overexpression of the 180 kDa isoform which is in good agreement with the putative role of this isoform in the maintenance of stable contacts in terminally differentiated neuronal cells.

ASTROCYTES IN THE MOTOR SENSOR CORTEX DURING STIMULATION OF BRAIN STRUCTURES

We-135 R.A. Chizhenkova

Institute of Cell Biophysics, Acad. Sci. Russia, Pushchino, Moscow Region, Russia

In unanesthetized unrestrained rabbits the stimulation (frequency 2/sec) of the medial lemniscus, the midbrain tegmental reticular nucleus, and the pyramidal tract produces neuroglial reactions in deep layers of the sensorimotor cortex. The stimulation during 1.5 hr causes a statistically significant increase of the number of revealed astrocytes. The same stimulation during 5 min on the contrary decreases the amount of these glial cells. The aforesaid neuroglial reactions are of a functional character.

We-136 THE QUANTITATIVE METHOD FOR INVESTIGATIONS OF BRAIN ENDOTHELIAL CELL POPULATION IN SITU
N. Lyubimova,

Institute of Theoretical and Experimental Biophysics of RAS, Pushchino, Moscow Region, 142292 Russia.

The investigation of time-dependent changes of brain endothelial cell population in normal and tumor tissues is a serious problem in cell biology. For the quantitative estimation of the endothelial cell number a fluorescent-histochemical technique was developed. It is based on the ability of the brain endothelial cell population to accumulate biogenic amines after pharmacological treatment of the animals. The rats were killed, the brain was dissected, immediately frozen in liquid nitrogen and lyophilized at a low temperature. Alternatively, one can use the method of low-temperature dehydration of frozen tissue samples by substituting liquid propane for ice. The luminous spots along the vessel walls as cell markers stand out sharply against the dark background. Electron microscopy was used to locate monoamine luminous spots in the endothelial cells. The number of cells and its linear density in the vessels were analyzed in brain section. A good agreement between the measured parameters and available literature data obtained by other method confirms the adequacy of our method for investigation of normal and pathological states of endothelial cell populations.

We-137 DEVELOPMENTAL CHANGES IN THE EXPRESSION AND COMPARTMENTALIZATION OF THE GLUCOCORTICOID RECEPTOR IN THE EMBRYONIC RETINA

L. Ben-Dror, R. Gorovits, R. Grossman, L. E. Fox, and L. Vardimon
Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel

Trans-activating capability of the glucocorticoid receptor (GR) increases markedly during development of chick retina, although the amount of GR protein does not change greatly. This apparent discrepancy was investigated by examining the pattern of GR expression. Two GR isoforms, 90 and 95kDa, were found to be expressed in the embryonic retina at a similar total amount but at different relative quantities. In early retina the 90 kDa receptor was higher whereas in late retina the 95kDa receptor was higher. These differences diminished upon addition of hormone which caused a rapid decline in the level of the 90kDa receptor and an increase in the nuclear level of the 95kDa isoform at both ages.

Using the gliotoxic agent D,L-alpha aminoacidic acid, which can selectively destroy glial cells, we demonstrated that in the differentiated tissue GR expression is predominantly restricted to Müller glia. This finding was confirmed by immunohistochemical analysis which also revealed a marked change in receptor localization during development. While in the differentiated tissue GR expression was restricted to Müller glial cells, in the undifferentiated retina GR was expressed in virtually all cells. Müller glia represent approximately 20% of the cells in this tissue and are the only cells in which glucocorticoid hormone induces the glutamine synthetase gene. Because the total amount of the receptor protein did not alter with age, its restriction to Müller glia implies a cell-type specific increase in receptor expression. We suggest that compartmentalization of GR in Müller glia is a major aspect of the mechanism that modulates receptor activity during development and results in the temporal increase in the inducibility of glutamine synthetase and its specific localization in Müller glial cells.

ADHERENT CELLS IN DISSOCIATED BRAIN CELL CULTURES

We-138 D. Kadiysky and M. Svetoslavova

Institute of Cell Biology and Morphology,
Bulg. Acad. Sci., G. Bonchev str. 25, 1113 Sofia, Bulgaria

The quick adherence to glass surface is a privilege of many cell types of the mononuclear phagocytic system. In CNS this system is represented by ED2-positive peri-vascular cells, exogenous macrophages, meningeal monocytes, many other cells, accidentally concentrated in the brain during different pathologic states, and probably by microglial cells. By virtue of the fact that these populations express MHC (major histocompatibility complex) antigens they are thought to be involved in the antigen presentation process in CNS.

It is well known that after 7-10 days in vitro dissociated mixed brain cells enrich in neurons or in glia, depending on the specific conditions of cultivation. We studied the dynamics of the adhering cells soon after the beginning of cultivation of the dissociated adult mouse brain cells. The initial ability for adhesion to glass was estimated 24, 48 and 72 h after culture seeding. The cell type of the adhering cells was verified by electronmicroscopy.

The spreading of the adherent cells increases during the cultivation. The attached to glass adherent cells do not possess essential phagocytic capacity. After 48 and 72 h of cultivation there are two different types adherent cells, forming clusters or linear forms. Light-microscopic observations reveal clusters from several rounded non-spread cells attached to a centrally situated flat polygonal cell. The adherent monolayer contains the following cell types, as demonstrated by EM, macrophages, fibroblasts large number microglial and some astroglial cells.

INTRACELLULAR pH AND MEMBRANE POTENTIAL ARE DECREASED BY HEAT SHOCK PROTEIN INDUCING TREATMENT IN C6 RAT GLIOMA CELLS APPLICATION OF IMPROVED IN VIVO NMR AND FLUORESCENCE TECHNIQUES:

Sören Skrandies¹, Ulrich Neuhäus-Steinmetz², Ulrich Pilatus¹
Ludger Rensing², and Adalbert Mayer¹

¹Institute of Experimental Physics, ²Institute of Cell Biology, Biochemistry and Biotechnology
University of Bremen, Germany

Intracellular pH (pH_i) and membrane potential were measured during heat shock, ethanol and paracetamol treatment in C6 rat glioma cells. For the measurement we developed temperature regulated continuously perfused *in vivo* ^{31}P -NMR and fluorescence spectroscopy methods. Both methods gave consistent results.

Heat shock caused a decrease in pH_i (approximately 0.4 units) and in membrane potential (approximately 30 mV). Ethanol (1.1 M) and paracetamol (80 mM) caused nearly the same changes in both parameters when applied in heat shock protein (HSP) inducing concentrations.

These results show that pH_i and membrane potential are involved in the heat shock response and may play a role in the induction mechanism, which is to date not yet fully understood.

ALPHA2MACROGLOBULIN RECEPTOR EXPRESSION IN HUMAN NEUROBLASTOMA CELL LINES.

We-140 Businaro R.*, Fabrizi C.* , Lauro G.M.* , Starace G.* , Moestrup S.K* and Fumagalli L*,

*Dept. of Cardiovascular Sciences, "La Sapienza" University Rome; **III University-Rome; ***Institute of Experimental Medicine, CNR, Rome, Italy; ****University of Aarhus, Denmark.

Alpha2 Macroglobulin Receptor (A2M-R) is formed by a membrane-spanning 85-KDa β chain and an extracellular 500-KDa α chain including binding sites for activated α 2M and apolipoprotein E. Receptor-mediated endocytosis and lysosomal uptake of the α 2M proteinase complexes have been demonstrated. Besides its ability to inhibit a wide range of proteinases α 2M interacts with several cytokines, including NGF, IL-1 and IL-6 suggesting a potential role in the regulation of their activity. Previous results showed that α 2M is produced *in vitro* by glial cells of neoplastic as well as embryonic origin and A2M-R is present at the level of neurons and glia in adult human brain sections. In the present paper we demonstrate the expression of A2M-R in a panel of human neuroblastoma (NB) cell lines. Confocal microscope immunofluorescence studies using monoclonal antibodies directed against A2M-R revealed that many cell lines bear the A2M-R in their plasma membrane. These results were confirmed by immunoblots and by flow cytometric analysis which suggests a uniform, even if relatively low, expression of the A2M-R in all cells of positive NB cell lines. Experiments are now in progress to correlate the A2M-R expression with the NB cells differentiation and to evaluate a possible modulation of A2M-R by various inducers.

OUTGROWTH IN VITRO OF ADULT FROG
SENSORY AXONS IN MATRIGEL

We-141 M. Edbladh, M. Kroon and A. Edström

Department of Animal Physiology, University of Lund,
Helgonavägen 3B, S-223 62 Lund, Sweden

By the use of a new method, which employs Matrigel as a growth substrate, some early phenomena occurring prior to and during the initial outgrowth of adult frog sensory axons in vitro were studied. The present in vitro preparation allows pharmacological investigation of biochemical events in the ganglion, the nerve and/or the naked regenerating axon.

Frog DRGs with 5 - 10 mm of their attached peripheral nerves and roots were dissected, covered with Matrigel and cultured in a modified RPMI 1640 medium at 20° C. Axons started to grow from the dorsal root and from the nerve ending into the gel within 2 days and continued elongating at a maximum rate of 6 $\mu\text{m}/\text{h}$ for several days. After about 5 days, non-neuronal cells began to migrate into the gel.

In preparations where the peripheral nerve ending, but not the ganglion, was continuously exposed to cycloheximide there was no axonal outgrowth. This seemed to be due to a local inhibitory effect since outgrowth from the dorsal root was not affected. These results stress the importance of proteins locally synthesised at the site of injury for the initial stages of axonal outgrowth.

THE ERK KINASE IS RAPIDLY AND
STRONGLY INCREASED IN THE

We-143 REGENERATING MOUSE SCIATIC NERVE

B. Svensson and P.A.R. Ekström,
Dept. Animal Physiology, Univ. Lund, Sweden

The extracellular signal regulated kinase ERK has recently attracted a lot of interest, and appears to be involved in various cellular functions related to growth and proliferation. However, little is known about its presence and role in the cells of peripheral nerves.

In the present study we have looked for the ERK1 protein in the mouse sciatic nerve and for possible changes of its expression in relation to nerve regeneration. In the normal nerve ERK is present in a small number of the nerve cell bodies in the dorsal root ganglion, more strongly so in the nucleus than in the cytoplasm, but also in many of the quiescent Schwann cells in the nerve trunk. Following a local injury the staining intensity as well as the number of positive nerve cell bodies is rapidly (<24h) and robustly increased. Likewise the Schwann cells in the vicinity of the injury show a strong increase in ERK immunostaining.

Other cell types with positive reaction include certain endothelial cells and leukocytes, the latter either resident in the nerve or recruited to the injured area from the blood stream.

The results indicate that the ERK kinase has important functions in cell growth and proliferation in the regenerating peripheral nerve.

PROTEIN KINASE C IS INCREASED IN

We-142 THE IN VITRO REGENERATING MOUSE
SCIATIC NERVE

P. Wiklund, P.A.R. Ekström and A. Edström,
Dept. Animal Physiology, Univ. Lund, Sweden

The mechanisms that initiate and regulate peripheral nerve regeneration are not known in great detail, but are likely to include various protein phosphorylating systems. Protein kinase C (PKC) is particularly abundant in nervous tissue and constitutes a family of at least 9 different isozymes, whose individual localizations and functions are unknown.

By the aid of immunohistochemistry we now report that the PKC- β isozyme is upregulated in the sensory nerve cell bodies as well as in the Schwann cells close to the lesion in the crush-injured, in vitro regenerating mouse sciatic nerve. The increase, which occurred within the first 24 h of culturing, involved both increased staining intensity and number of PKC- β positive nerve cell bodies and Schwann cells. The staining of the nerve cell bodies was strictly cytoplasmic and there was also sign of axonal transport of the enzyme.

In parallel experiments specific PKC antagonists were found to have diverse effects on nerve regeneration and the injury-induced proliferation of Schwann cells.

In conclusion, our results suggest that PKC has a role in the mechanism of peripheral nerve regeneration and that this at least in part might be brought about by the PKC- β subtype.

MACROPHAGES AND REGENERATION OF THE
RAT VAGUS NERVE IN VITRO AND IN VIVO

We-144 M. Kanje and S. Magnusson
Department of Animal Physiology, University of Lund,
223 62 Lund, Sweden

Macrophages have been suggested to play multiple roles during regeneration of peripheral nerves. Following nerve lesions, macrophages invade the nerve to participate in Wallerian degeneration. In addition, macrophages secrete factors which affect synthesis of neurotrophic protein in Schwann cells. Consequently, regeneration is enhanced by factors which favours macrophage recruitment. However, we have found that the adult vagus nerve of the rat regenerates in culture at a velocity close or similar to that in vivo. This must occur in the absence of invading macrophages since no such cells can be recruited to the nerve during the culture period. It was therefore of interest to compare the macrophage content of regenerating vagus nerves in vitro and in vivo.

Antibodies to ED1 and ED2 which reacts with invading and resident macrophages respectively were used to immunostain sections of 1-3 days regenerating rat vagus nerves. In addition some sections were stained for the presence of NGF receptors.

The nerve contained many resident macrophages as revealed by ED2 staining. However their number did not appear to increase significantly following a nerve lesion either in vitro or in vivo. The staining pattern of ED1 positive cells, as anticipated, differed between the in vivo and in vitro preparations. Numerous ED1 positive cells were observed at the crush lesion but also distal to the lesion in vivo. Their numbers increased with time. In contrast very few positive cells were observed in the in vitro preparations but some staining unrelated to macrophages was observed in the crush region. The NGF receptors showed a dramatic increase at and distal to the lesion in vivo and along the entire nerve in vitro.

The results suggest that invading macrophages are not required for the initial and early regenerative response in peripheral nerves.

**ISOLATION OF N-ACETYL-D-GALACTOSAMINE
CONTAINING GLYCOPROTEINS FROM RAT
CENTRAL NERVOUS SYSTEM**

We-145

I. Fakla, A. Molnar, J. Fischer

Albert Szent-Györgyi Medical University, Department of
Biochemistry, Szeged

Glycoproteins play an important role in cell-cell interactions, partly as receptors for endogenous lectins or as immunodeterminant groups.

The introduction of exogenous (plant or animal) lectins in biological studies allowed the detection and isolation of some individual glycoproteins. The association constant of lectins to oligosaccharide chains of glycoproteins, is generally between 10^7 to 10^{10} M^{-1} , comparable to antigen-antibody complexes.

Previous histological studies showed that some cell types of the rat central nervous system produce glycoproteins in small quantities. The aim of this study was to characterize and isolate these glycoproteins by means of biochemical methods.

Lectins, specific to N-acetyl-D-galactosamine from soybean (SBA) and Vicia villosa (VVA), mannose from Canavalia ensiformis (ConA) and poli-N-acetyl-lactoseamine from tomato (TL) were used. An endogenous lectin of human brain (HBL) were also used. Western blotting revealed three lectin binding glycoproteins in the supernatant (100000g) of the homogenate without any detergent, with molecular weights of 122, and a double band at about 77kD. Dissolving the membrane fraction in detergents (ZWITTERGENT or TRITON X-100) resulted in one more band of 183kD which reacted with TL only. SBA, VVA and HBL lectins showed similar results but the ConA has different specificity. After affinity chromatography on VVA-Sepharose column only the 122kD band was specifically eluted. PAGE of the lectin affinity purified material indicates that the low molecular weight fractions might be aggregated to a 180-200kD band, which however loses its lectin binding capability.

**TOMATO LECTIN: MARKER OF AN ASTROCYTE
SUB POPULATION IN RAT CENTRAL NERVOUS
SYSTEM**

We-146

J. Fischer^a, I. Fakla^a, Cs. Torday^b and L.

Latzkovits^b

^aDepartment of Biochemistry and ^bDepartment of Experimental Surgery, Albert Szent-Györgyi Medical University, H-6701 Szeged, P.O.Box 415, Hungary

By means of labelled tomato lectin (TL) a small group of glial cells were intensively stained in almost all areas of adult CFY rat brains. The staining intensity varied from faint to high density labelling. The most intense staining was seen in the median eminence and around the third ventricle. Cell bodies and the ramified glial processes were equally stained. The glial processes often formed nets around neurones. In the cerebral cortex the lectin reactive glial envelope was mainly seen around basket cell type neurones, while in the spinal cord glial nets were seen around both dorsal and ventral horn neurones. Lectin labelling was seen along the Ranvier nodes of myelinated fibres in the dorsal roots, too. Lectin stained glial processes formed contacts with the capillary network as well.

According to their distribution and morphological appearance the TL reactive glial cells are astrocytes. Their number, however, is far from the total astrocyte count of the CNS. A sub population of astrogliia is labelled only, which apparently forms a bridge between some glycoproteins synthesising neurones and the likewise TL binding endothelia. To emphasise the morphological findings astrogliial cell cultures were also studied. GFAP proven astrocytes of three month cultures showed intense intracellular staining with the TL, too. Western blot analysis of cultured astrocyte cell homogenates, but not that of the culture medium, showed identical glycoprotein bands with glycoproteins from homogenates of the CNS.

**IMMUNOCYTOCHEMICAL LOCALIZATION OF
Cu,Zn-SUPEROXIDE DISMUTASE IN RAT
BRAIN**

S. Moreno, M. Giorgi, A.M. Cimini and
M.P. Ceru

Dept. of Basic and Applied Biology -University
of L'Aquila- ITALY

The distribution of Cu,Zn-SOD in rat brain was examined by immunocytochemistry and immunoelectron microscopy, using affinity purified antibodies. The enzyme appears exclusively localized in neurons; the intensity of staining is variable depending on the examined brain region and, within the same area, on the neuronal type considered. Immunoelectron microscopic observations on the reticular thalamic nucleus confirm the specificity of Cu,Zn-SOD for neuronal cells, as no staining is visible in non neuronal cells. Immunostaining is seen both in the cytosol and in the nucleus of small neurons and it extends from their somata to their dendrites and axons. Moreover, the immunoreaction product often appears concentrated in membrane-limited organelles resembling peroxisomes. Our results suggest a selective expression in nervous cells of different antioxidant enzymatic system, depending on the cell type (neuronal/non-neuronal) and on the different neuronal populations. Moreover, our data seem to support the hypothesis that in nervous tissue Cu,Zn-SOD, besides being located in the cytosol and in the nucleoplasm, is also present in peroxisomes.

**INDUCTION BY RETINOIDS OF THE ACTIVITY OF
TYPE III-DEIODINASE RESPONSIBLE FOR THY-
ROID HORMONE DEGRADATION IN ASTROCYTES**

A. Esfandiari^a, C. Gagelin^a, J.M. Gavaret^a, S.
Pavelka^{b,c}, A.M. Lennon^a, M. Pierre^a, F. Courtin^a.

^aINSERM (U.96) 80, rue du Gal Leclerc 94276 Kremlin-Bicêtre
cedex France; ^bInstitute of Physiology, Academy of Sciences, 14200
Praha, Czech Republic; ^cDepartment of Biochemistry, Faculty of
Science, Masaryk University, 61137 Brno, Czech Republic.

Thyroid hormones and retinoic acid (RA) are potent modulators of growth, development and differentiation. Type III deiodinase (D-III), which catalyzes thyroid hormones degradation in the brain was shown to be induced in cultured astrocytes by multiple pathways, including cAMP, TPA, fibroblast growth factors and thyroid hormones themselves. In the present study, the effects of retinoids on D-III activity were examined in astrocytes.

Cells were obtained from the cerebral hemispheres of 2-day-old rats and grown in DMEM medium containing 10 % fetal calf serum. Confluent cells (about 10 days) were further cultured in a serum-free defined medium (DMEM/F12) for 5 to 6 days. Incubation of astrocytes with 5 μM all-trans-RA caused up to 200-fold increase in D-III activity, which reached a plateau after 48 h. The retinoid-induced increase in D-III activity was reversible and concentration-dependent (0.5 μM all-trans-RA and 9-cis-RA producing half-maximal effect). Retinol was effective at physiological concentrations (1 and 10 μM).

The potent action of retinoids on the enzyme responsible for thyroid hormones degradation in the brain may protect the brain from the effects of thyroid hormones in regions influenced by retinoids.

We-149

QUANTITATIVE ASSESSMENT OF THE TIME COURSE
AND EXTENT OF GFAP IMMUNOREACTIVITY
ENHANCEMENT ACCOMPANYING LESION INDUCED
REMOTE ASTROCYTIC RESPONSE

V. Jancsik, P. Sótonyi and F. Hajós

Department of Anatomy and Histology,
University of Veterinary Science, Budapest, HUNGARY

Earlier immunohistochemical studies demonstrated time dependent increase of GFAP immunoreactivity in the primary visual cortex of the rat as a result of unilateral stereotaxic destruction of the dorsal lateral geniculate nucleus. This was interpreted in terms of a remote astroglial response induced by retrograde degeneration of afferent neurons [Hajós et al., (1990) Glia 3: 301-310].

Quantitative follow-up of these studies will be presented here. GFAP content of the ipsilateral (impaired) and contralateral (control) sides of the primary visual cortex was compared by western blot technique followed by densitometric evaluation.

Significant enhancement of the GFAP immunoreactivity difference between the two sides was observed on postoperative days 7. and 14., whereas four weeks after the operation this difference was no longer apparent.

These results lend further support to the biphasic nature of remote astrocytic response and speak for an important role of the glial intermediate filaments in this process.

This work has been supported by the Hungarian Academy of Sciences (Grant OTKA no. 1245)

We-151

CORTICO-PIAL PERIVASCULAR SPACE.
AGE DEPENDENCE OF THE STRUCTURAL
HOMEOSTASIS.

E.TEDESCHI, P. Crafa, N. Campanini, G.R. Trabattoni*

Department of Pathology and Neurology* -
University of Parma - ITALY

An EM and immunocytochemical study of the cortico-pial space in the human was performed, to evaluate possible age related changes. Cortico-meningeal samples of normal pressure patients from the 7th to the 9th decade of age were considered. Cerebral samples from patients who were hypertensive and with arteriosclerosis were used as control. Immunocytochemical analysis was based upon the following antigens: GFAP, EMA, NSE, F-VIII, Neurofilaments, Actin, S-100, Fibronectine, Laminine. In the elderly, the cortical glial limitans of the brain preserved continuity of extension, but resulted thickened up and fibrous. The thickening was more remarkable in correspondance with the cortico-pial perivascular spaces (of Virchow-Robin), due to specific positivity for the GFAP. Also the related sub pial basal lamina denoted thickening, at times in the presence of amyloidous bodies along the face towards the cortex. In this location the cytoplasmic expansions of the astrocytes would sometimes be swollen being their matrix devoid of structure, and with variable electron density. On the arachnoid slope the meningocytes presented with their typical plasmalemma endowed with numerous desmosomes; an outstanding fibrillary collagen amount was noted. The small arteries that deepened into such space had perivascular lumen and regularly formed wall; the contiguous small arteries and venules underneath the arachnoid presented with the same features. After these findings, we conclude that in primary senescence of the human the age related changes do not impair the structural homeostasis of the cortico-pial perivascular space.

We-150

THE ROLE OF GLIA IN IONIC AND VOLUME
HOMEOSTASIS IN CNS
E. Syková, P. Jendelová, J. Svoboda, A. Chvátal
Institute of Experimental Medicine, AVCR,
142 20 Prague, Czech Republic

Glial cells control ionic homeostasis and extracellular space (ECS) diffusion parameters. Activity-related changes in extracellular K⁺ concentration ([K⁺]_e), pH (pH_e) and the ECS volume fraction (α), tortuosity (λ) and nonspecific cellular uptake (k') - three parameters affecting the diffusion of substances in the CNS - were studied in rat spinal cord, cortex and corpus callosum (CC) using ion-selective electrodes. Concentration-time profiles of TMA⁺ applied by iontophoresis were quantitatively analyzed.

Stimulation of an afferent input elicited a rise in [K⁺]_e and acid change in pH_e. In animals at P3-7, the K⁺ ceiling level was elevated, and there was an alkaline shift (AS). The decrease in the [K⁺]_e ceiling level and the acid shift in pH_e occurred at P10-14^o and were blocked by X-irradiation, the procedure which blocks gliogenesis. The AS was blocked by Mg²⁺, picrotoxin and glutamate receptor antagonists, the acid shift by acetazolamide and Ba²⁺. This suggests that the acid shifts are related to membrane transport processes in mature glia, while the AS have a postsynaptic origin and result from a HCO₃⁻ efflux or a H⁺ influx through ligand-gated ion channels.

The ECS space in the adult CNS occupies about 20% of the tissue, $\alpha=0.20\pm0.003$, $\lambda=1.62\pm0.02$, $K'=4.6\pm0.4\times10^{-3}s^{-1}$ (mean \pm SE). In gray matter at P3-7 and in CC at P3-11 the α was about double that of adults. In adults stimulation or injury evoked a shrinkage of the ECS by 20-50%, while no shrinkage was found at P3-6. It is evident that the shrinkage is related to swelling of mature glia. In rats with EAE or after X-irradiation the α was about double of that in the controls, while during anoxia the α rose to 0.05 ± 0.006 , and λ fell to 2.20 ± 0.07 . Changes in the ECS diffusion parameters may aggravate accumulation of ions, transmitters, growth factors, neuropeptides, metabolites etc., and so alter development, non-synaptic transmission, or contribute to the CNS damage.

We-152

GFAP GENE EXPRESSION DURING POSTNATAL
DEVELOPMENT

M. TARDY, T. LEFRANCOIS, C. FAGES, G. LE PRINCE
Inserm, U-282, Hôpital Henri Mondor, 94010 Creteil,
France

GFAP is the major component of the astroglial intermediate filaments. It is considered as both a differentiation marker and an index of astroglial reactivity.

During astroglial maturation, most of the GFAP-mRNA transcripts appear during the early perinatal period. This early transcription is associated with a rapid turnover of the encoded protein (half-life=16-18 hours). At that time, GFAP appeared as a polymerized network close to the cell nuclei and the centriolar region. GFAP-mRNA steady-state levels, measured by Northern blot, increase during the proliferating state. But while astrocytes change their shape and stabilize their processes, the message level decreased. This diminution was correlated with the drop of the amount of transcripts and consistent with a transcriptional control during this period. A stabilization of the GFAP is supported by a low turnover rate (half-life=5-6 days). During that time, astrocytes develop numerous processes, in which both GFAP and GFAP-mRNA are expressed. A role of GFAP in the early mitotic control may be hypothesized. GFAP is at minima involved in the stabilization of the astroglial cell-shape in a given environment. Changes in GFAP expression in physiopathological conditions, may modify signalling transduction and related events and also cell to cell interactions. An antisense GFAP-mRNA strategy is under investigation in order to understand better the role of this protein during the cell division and differentiation.

We-153 THE CELL CYCLE RESPONSE OF NORMAL & TRANSFORMED GLIAL CELLS TO CIS-DICHLORODIAMINE PLATINUM (*cis*-DDP)

V. Mares ^{a,c}, V. Lisa ^a, G. Mazzini ^b, R. de Renzis ^b and F. Kocourek ^b

^a Institute of Physiology, Czech Academy of Science, Prague;

^b Centro di Istitochimia, C.N.R., Pavia and ^c Department of Zoology, Kuwait University, Kuwait City

Transformed C6 glioma cells in culture and normal cells of the immature cerebellum and thymus in 6-day-old rats *in situ* were exposed to *cis*-DDP (1 to 5 µg/g b.w.) and examined by DNA flow-cytophotometry 8 to 72 h later.

The first response of normal cells appeared at 8 h and was represented by arrest of a large part of the population in G1; the cells which escaped this block were trapped at G2/M phase by 24 h. The response of the C6 glioma cells was delayed (24 to 72 h) and the main part of the population was stopped at G2/M. Cell death in the C6 population became apparent after 72 h. This was much later than in the normal cells *in situ* (8 to 24 h, Mares et al. Exp. Neurol. 91, 246, 1986). C6 glioma cells grown at lower population densities were found more sensitive to *cis*-DDP treatment; a small number of cells in these cultures responded even by an early G1/S block. According to the preliminary data, the response of the normal immature glial cells grown culture is similar to that in normal cells grown and treated *in situ*. The G2/M block of C6 glioma cells could be attenuated by culturing cells in the presence of EGTA.

The data revealed a weaker control of the cell cycle in G1 in the C6 glioma cells; efficiency of this control point was found relatively lower in cells grown at higher densities. (The study was supported by the Grant of the Czech Academy of Science No. 71105).

NEUROTRANSMITTER RECEPTORS IN GLIAL CELLS

We-154 H. Kettenmann

Max-Delbrück-Center for Molecular Medicine, Department of Neurobiology, Berlin, Germany

We have used brain slices to characterize membrane channels and receptors in glial cells from an intact tissue. In this presentation, pharmacological and biophysiological properties of glial receptors will be described and their properties will be compared to the ones expressed in neurons. Astrocytes and oligodendrocytes have distinct GABA and glutamate receptors which can be linked to Ca²⁺ signalling. The receptors and the subsequently activated "second messenger" cascades are distinct in the different types of glial cells. Glial receptors might play a role in synaptic transmission and speculations in this direction will be discussed.

POTENTIAL NON-SENECENCE OF HUMAN CELLS AND HUMAN BODY

We-155 A. Khalyavkin

Department of Kinetics of Chemical and Biological Processes, Institute of Chemical Physics, Russian Academy of Sciences, Kosygin Street 4, Moscow, Russian Federation

The facts says: the anti-aging processes can successfully counteract cellular deterioration, and loss of the mitotic activity *in vitro* is a manifestation of differentiation but not of the aging. Why does an organism hypothetically composed of potentially non-senescent cells age? Because the effectiveness of self-maintenance may depend not only on structural/functional peculiarities of an organism but also on the conditions in which it exists. A potentially non-senescent organism is able to sustain a physiological regimen of complete self-maintenance only within a certain range of changes in external conditions. Outside the optimal zone organism will begin to age. This arguments are supported by the correlation between parameters of death statistics for different countries which is similar to the death pattern for populations of potentially ageless organisms, which age in conditions preventing the complete self-maintenance of an organism. Both realization of this fact and understanding that the human body consists of potentially non-senescent cells make it possible to embark on a search for living conditions and physiological regimens which would reduce senescence as far as desirable.

INTERCELLULAR INTERACTIONS IN RADIATION-INDUCED APOPTOSIS OF THYMOCYTES

We-156 O. Dobrovinskaya, V. Shaposhnikova, L. Eidus, Yu. Korystov

Institute of Theoretical and Experimental Biophysics of RAS, Pushchino, Russia

Radiation is known to induce the apoptosis in thymocytes. A similar situation takes place in the course of negative selection of cortical thymocytes by thymus microenvironmental factors. That is the reason why intercellular interactions in apoptosis have been in the focus of attention in this study. Subpopulation analysis points to the fact that natural killer cells, mature thymocytes and macrophages have no influence on the damage progression in irradiation (6 Gy) cortical thymocytes. At the same time lowering of the cell concentration, mixing the cell suspension, changing the incubation medium as well as postirradiation incubation of cortical thymocytes together with unirradiated thymocytes or with cells of certain cell lines decreased nuclear pyknosis and cell death estimated by trypan blue exclusion tests. Under these condition DNA fragmentation varied slightly or not at all affected. On the other hand, thymocytes were shown to release 5-H-arachidonate from cellular membrane phospholipids during 30-60 min after irradiation, and arachidonic acid (AA) metabolism inhibitors blocked DNA fragmentation as well as nuclear pyknosis. The cytosol from irradiated thymocytes (1h after irradiation) was cytotoxic for intact cells. It was suggested that some AA products may serve as local toxic mediators after irradiation.

ROLE OF PROTEIN KINASE C IN THYMOCYTE APOPTOSIS

We-157 V.Shaposhnikova, O.Dobrovinskaya,
L.Eidus, Yu.Korystov

Institute of Theoretical and Experimental Biophysics of RAS, Pushchino, Russia

It is shown that the protein kinase C inhibitor H7 (50 μ M) prevents the nuclear pycnotization of irradiated (6 Gy) rat thymocytes 6 h after incubation *in vitro* while the PKC activators such as phorbol 12-myristate 13-acetate (PMA 10 nM), calcium ionophore, A23187 (100 nM), concanavalin A (Con A, 5 μ g/ml) and arachidonic acid (50 μ M) stimulate the DNA fragmentation in thymocytes irradiated with a dose of 0.5-2 Gy. A species specificity in the reaction to the PKC activators has been revealed: PMA (10 nM) induced neither DNA fragmentation nor its stimulation in irradiated (0.5 Gy) rat thymocytes, as opposed to mouse thymocytes. The measurements of $[Ca^{2+}]_i$ using quin-2 have shown that its level does not change in thymocytes after irradiation with a dose of 6 Gy, indicating that this way of PKC activation is not realized in these conditions. Another way of PKC activation was found to be realized through phospholipase products. We were able to show that the inhibitor of phospholipases BFB (3 μ g/ml) reduced the level of DNA fragmentation in thymocytes under the action of the PMA, Con A, A23187, and their combined action with irradiation to the control level. The data obtained point to the participation of PKC in the postirradiation apoptosis of thymocytes and to a possible role of phospholipase products in its activation.

STEPWISE DNA DEGRADATION IN POSTMITOTIC DEVELOPMENT AND APOPTOSIS OF WI-38 FIBROBLASTS

We-159 Klaus Bayreuther, Peter Brenneisen,

and Dietmar Mayer

Institut für Genetik, Universität Hohenheim,
D 70599 Stuttgart FRG

WI-38 fibroblasts develop along a seven stage differentiation sequence of mitotic fibroblasts MF I-II-III and postmitotic fibroblasts PMF IV-V-VI-VII. PMF VI is the terminally differentiated end cell of the fibroblast stem cell system. PMF VII degenerates by apoptosis. The genomic DNA of MF WI-38 is made up of large chromosome double-strand(ds) fragments of about 1.9 mb, that of early PMF WI-38 of ds-fragments of 450-150 kb (chromatin rosettes 300 kb), and that of late PMF WI-38 of ds-fragments of 225-100 kb (chromatin loops 50 kb). In the same period of development the length of the single-strand (ss) DNA-fragments of PMF WI-38 decreases to 20 kb. The 2-D-PAGE pattern of (35 S) methionine labelled polypeptides of early and late PMF WI-38 is identical, indicating that the DNA-degradation down to the observed level is a normal process in the postmitotic development of WI-38 cells. Late WI-38 PMF can be experimentally induced to go into apoptosis, in apoptosis the DNA is cleaved at the internucleosomal level. The same sequence of ds-and ss breaks and cleavage at the internucleosomal level takes place within 6-8 h, when MF WI-38 are experimentally induced to enter apoptosis.

TRANSCRIPTION FACTORS IN APOPTOSIS OF RAT THYMOCYTES.

We-158

E. Radziszewska*, E. Sikora*, E. Grassilli, E. Bellesia and C. Franceschi.

*Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, POLAND.

Department of Biomedical Sciences, University of Modena, Via Campi, 287, 41100 Modena, ITALY.

The onset of apoptosis is generally thought to require the induction of novel genetic program. The first step of gene regulation depends on proteins named transcription factors (TFs). Accordingly, we studied the kinetic of DNA-binding activity of several transcription factors in rat thymocytes undergoing apoptosis induced by dexamethasone (DEX) or heat shock (HS) treatment. Here we report that: 1) early activation of AP-1 occurred in both models of apoptosis, even if the intensity of activation and AP-1 complex composition were different and much less evident in HS-treated thymocytes; 2) early NF- κ B DNA-binding activity was also observed in both types of apoptosis; 3) downregulation of other transcription factors, such as OCT-1 and CREB, with high constitutive activity, was recorded in both models of apoptosis. The fact that some TFs are up-regulated while others are down-regulated suggest that apoptosis is the result of a complex combination of positive and negative signals regulating gene expression.

DOWN-MODULATION OF CD4 CAN BE TRIGGERED BY PROGRAMMED CELL DEATH

We-160

W. Malorni, R. Rivabene, G. Rainaldi, M.T. Santini, and G. Donegli

Department of Ultrastructures, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

It has been hypothesized that programmed cell death (PCD), an active cell suicide process occurring in alternative to necrosis, can be associated with the pathogenesis of acquired immunodeficiency syndrome (AIDS). The entry of human immunodeficiency virus (HIV) into competent cells is mediated by the CD4 molecule present on the surface of certain lymphocyte subpopulations as well as on some cultured cell lines, e.g., U937 myelomonocytic cells. These cells are in fact used for the *in vitro* maintenance of HIV. The present report focuses on some specific aspects of PCD induced by the cytokine Tumor Necrosis Factor (TNF) on U937 cells. Particular emphasis was given to i) the role of cycloheximide (CHX), which activates the NF κ B factor and inhibits active protein synthesis, ii) the fate of the CD4 molecule during apoptotic cell death, and iii) the role of intracellular oxidative imbalance during these phenomena. The results indicate that the exposure of U937 cells to cycloheximide is able of "facilitating" the occurrence of TNF-mediated PCD via a short term cell death program as well as modifying the expression of CD4 surface molecules. Moreover, an analysis of other plasma membrane parameters, such as membrane order or the absence of variations in the expression of other surface molecules such as CD3 and CD71, was also carried out. Finally, considering the role of oxygen free radical production in TNF subcellular activity and its relationship with PCD, we examined the possibility of counteracting these complex events by using the antioxidant N-acetylcysteine (NAC). Our results indicate that NAC pretreatment was able of preventing PCD, but not the CD4 internalization as consequence of CHX/TNF exposure.

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RETINOIC ACID REGULATION OF APOPTOSIS IN
NEURAL CRESTS-DERIVED CELLS: ROLE OF
C-MYB AND "TISSUE" TRANSGLUTAMINASE

M. Piacentini

Dept of Biology, University of Rome "Tor Vergata" Italy.

Human neuroblastoma (NB) cells in culture comprise distinct cell types which recapitulate neural crest cells development. One type is neuroblastic (N) with characteristics of noradrenergic neurons, another prominent cell type is substrate-adherent (S) which expresses marker proteins for schwannian/glial/melanocytic cells. We investigated the cellular and molecular mechanisms of retinoic acid (RA)-induced cell death in relation to the phenotypes expressed by human NB cells. "tissue" transglutaminase (tTG) gene is specifically expressed in apoptotic cells. The Ca^{+2} -dependent activation of this enzyme leads to the formation of cross-linked protein polymers which stabilize the dying cell before phagocytosis. Overexpression of tTG in the NB renders these cells highly susceptible to death by apoptosis. In fact, SK-N-BE(2) NB cells transfected with a full length tTG cDNA display a drastic reduction in their proliferative capacity paralleled by a large increase in the cell death rate. The dying tTG-transfected cells show the cytoplasmic as well as the nuclear changes characteristic of apoptotic cells. The tTG-transfected cells show a drastic reduction in tumor progression capacity when injected in SCID mice when compared with the parental cells. In addition, transfection of NB cells with segments of the human tTG cDNA in antisense orientation resulted in a pronounced decrease of both spontaneous as well as RA-induced apoptosis. We also show that the apoptotic program of NB cells is strictly regulated by retinoic acid and that apoptosis in SK-N-BE(2) cells preferentially occurs in the S-phenotype. These results demonstrate that tTG expression is a key event in the maturation toward apoptosis; thus suggesting that cross-linking of intracellular protein may be responsible of the structural changes occurring in cells dying by apoptosis.

c-myb is expressed in NB in which is transcriptionally decreased by RA. Transfection of NB cells with *c-myb* cDNA in antisense orientation yielded fewer transfectant clones, with a slower proliferation rate than with sense orientation. The growth inhibition is enhanced by serum reduction to 1%, resulting in death of over 80% of the AS transfected cells. This dramatic arrest in growth is due to the induction of apoptosis which is accompanied by a net increase in tTG expression. However, FACS analysis indicated the absence of DNA fragmentation in the dead cells, suggesting an unusual pattern of apoptosis. These findings demonstrate that *c-myb* protein may act as a powerful negative controller of apoptosis in NB cells.

CANCELLED

We-162

A NOVEL BUTYRIC ACID PRODRUG INDUCES
APOPTOSIS IN PROMYELOCYTIC LEUKEMIC CELLS

Y. Zimra^a, M. Shaklai^a, and A. Rephaeli^b

^aFelsenstein Medical Research Center and Hematology Division, Beilinson Medical Center, Petah Tikva 49100 & Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; ^bAnsan Inc., 400 Oyster Point Blvd. Suite 315 S. San Francisco, Ca, 94080 U.S.A.

A novel butyric acid (BA) prodrug, pivaloyloxymethyl buturate (AN-9) was previously shown to exert significant anticancer activity in-vitro and in vivo. AN-9 acts at lower concentrations and faster than BA. The mode of action of AN-9 and BA is unclear: both induce histones hyperacetylation and modulate the expression of early regulatory and suppressor genes. Significant differences in the pattern of gene expression following treatment with low and high AN-9 concentrations was shown. This could reflect the differences in the cells' fate: at low concentrations, AN-9 induces differentiation whereas at high concentrations it causes cell mortality. Herein we show that AN-9 induces cell death by apoptosis. Apoptosis, an active form of cellular suicide, is manifested usually in the activation of endonucleases that degrade the cell DNA to oligomers of about 180bp. Exposure of HL-60 cells to 150 and 500 μ M AN-9 for 4h caused a decrease in cell viability at about 70% and the appearance of the typical apoptotic DNA "ladder" patterns after 24h. Similar results were obtained with 1500 μ M BA only after 48h. Exposure of the cells to AN-9 in the presence of the apoptosis inhibitor ZnSO₄ significantly restored cell viability.

We suggest that AN-9 and BA may exert their effects, at least in part, through the induction of apoptosis.

We-164

NEGATIVE GROWTH FACTORS FROM EHRlich
ASCITES CARCINOMA CELLS. IN VITRO TESTS.

F. Mainferme⁺, R. Wattiaux⁺, I. D. Postescu^{*}

and I. Mustea^{*,†}; Facultés Universitaires N. D. de la Paix, Namur, Belgium and ^{*}Oncological Institute, Cluj Napoca, Romania.

We have previously proved the versatility of time-controlled autolysis as a modality to obtain enhanced amounts of endogenous growth inhibitory factors from Ehrlich ascites carcinoma cells. Following the partial purification of crude lysates (extraction with 70 % ethanol solution and subsequent Sephadex G-25 fractionation), the major inhibitory activity was found, as measured in "in vivo" assays, in a pool containing low molecular weight components ($Mr < 1000$ Da). Preliminary analysis (color reaction, UV spectra, reverse phase chromatography) indicated the presence of peptides but the concomitant existence of other products whose possible link with the inhibitory activity could not be excluded. Here we report our "in vitro" investigations of the most active fractions obtained after G-25 fractionation of lysates, on the growth of Morris hepatoma 7777 and human acute lymphoblastoma leukemia Molt-4 cells. The growth rate of both cell lines was reduced by comparison with untreated cells, after incubation for 24, 48 and 72 h (at which time the effect was maximum) and presented a dose effect relationship in a relatively narrow range of concentrations. As, at high concentration, the fractions exert a cytotoxic activity, DNA was extracted from treated cells and analyzed by agarose gel electrophoresis to check if cell death occurs by an unspecific mechanism or by a more concerted process, as apoptosis.

CANCELLED

We-166

POST-TRANSLATION DEAMIDATION-CONTROLLED RATE OF PROTEIN CATABOLISM DURING AGING
A. Lukash, N. Puskina, I. Klimova,
I. Nasarova

Institute of Biology, Rostov-Don, Russia

Our experiments showed accumulation of deamidated proteins during aging and protein asparagine-dependent nonenzymatic autoproteolysis with increasing proteolytic attack of deamidated molecules. Deamidation leads to the partial decreasing of biological activity in different proteins (immunoglobulin, lysozyme, insulin, alcohol dehydrogenase).

The deamidation rate depends on nonenzymatic glycosylation of proteins by glucose. It may be reason of early aging at diabetes.

We-167

DETECTION OF APOPTOTIC DNA FRAGMENTATION BY *IN SITU* NICK TRANSLATION
L. Stuppia ^a, E. Falcieri ^{ab}, R. Peila ^a, S. Santi ^a, C. Cinti ^c, L. Zama ^d and N.M. Maraldi ^{a c}.

a. Istituto di Citomorfologia Normale e Patologica CNR di Chieti e Bologna
b. Istituto Morfologia Umana Normale Università di Chieti
c. Laboratorio Biologia Cellulare e Microscopia Elettronica c/o IOR Bologna
d. Istituto di Anatomia Umana, Università di Bologna (Italy)

Apoptosis is a controlled programmed cell death, which plays an essential role in a variety of cellular events (1). Recent reports demonstrated that the DNA fragmentation occurring during this process can be detected *in situ* by Terminal deoxynucleotidyl Transferase (TdT) (2). In this work we compared the results obtained with this technique with those produced by *in situ* Nick Translation (NT) involving the use of DNA polymerase I (Pol I) (3). Apoptosis was induced in HL60 human leukemia cells by 0.15 μ M camptothecin treatment for 6 h. Treated and control cells were fixed in acetic acid/methanol and submitted to TUNEL or *in situ* Nick Translation reactions and observed by confocal microscopy. Samples treated by *in situ* Nick Translation displayed a positive reaction, i.e. presence of DNA nicks in a higher percentage with respect to those treated by TUNEL, suggesting that the use of Pol I could improve the sensitivity of this kind of approach in the study of apoptosis.

- 1) Cohen J.J. (1993) *Immunol. Today* 14: 126-130.
- 2) Sgome et al. (1994) *T.J.G.* 10: 41-42
- 3) De la Torre et al. (1992) *Genome* 35: 880-894.

We-168

MODIFICATIONS OF CARDIOLIPIN CONTENT AND MITOCHONDRIAL MEMBRANE POTENTIAL DURING AGEING OF MURINE SPLENOCYTES AND HUMAN KERATINOCYTES.
M.H. Ratinaud*, A. Maftah*, M. Dumas**, F. Bonte**
* Institut Biotechnologie, Fac. Sciences- 87 LIMOGES;
** LVMH recherches- 92 COLOMBES- FRANCE.

In reason of their tight implication in cellular senescence, mitochondria phenotypic modifications during ageing were investigated from murine splenocytes (3 to 24 months) and women keratinocytes (9 to 75 years). For this purpose, flow cytometry and two mitochondrial fluorescent probes were used. Nonyl Acridine Orange (NAO) permits to quantify the cardiolipin, a specific marker of the inner mitochondrial membrane, while the uptake of Rhodamine 123 (Rh123) depend on the mitochondrial membrane potential ($\Delta\psi$).

In murine splenocytes, we observed that cardiolipin (CL) content remained stable until 12 months and then diminished by about 25% up to 24 months. On the contrary, the $\Delta\psi$ reduced nearly with age in animals older than 6 months to 50% of its initial level by 24 months. Thus the linear decrease of transmembrane potential occurred approximately six months prior the cardiolipin loss.

During ageing of women keratinocytes, CL content significantly declined ($r=0.540$, $p=0.002$) with a linear slope. The phenomenon was continuous from keratinocytes older than 9 years and led to a loss of 57% cardiolipin in keratinocytes older than 75 years. In contrast, the $\Delta\psi$ still unchanged ($r=0.04$, $p=0.6794$) for all patients (9 to 75 years).

Whatever cell origin, the amount of cardiolipin diminished, reflecting oxidative damages of mitochondrial structures. Alterations sustained by CL and mtDNA provoked respiration chain failures and therefore a disruption of ATP synthesis. For old murine splenocytes, the failure in mitochondrial respiration led to a lowering of $\Delta\psi$ (decline of Rh123 fluorescence); whereas for human keratinocytes mitochondrial membrane potential would really decrease with age but it would be compensated by glycolytic pathway (unchanged Rh123 fluorescence).

We-169

**ROLE OF THE MANNOSE SPECIFIC RECEPTOR
IN THE RECOGNITION OF APOPTOTIC CELLS**

M.T. Ruzlitzu, M.C. Vergine, A.P. De Palma,
A. Sozzo, M.R. Montinari, L. Falasca^a,
L. Conti Devirgiliis^a and L. Dini

Dept. of Biology, University of Lecce, Italy; ^a Dept. of Cellular and Developmental Biology, University of Rome La Sapienza Italy.

Apoptosis is a phylogenetically ancient process which plays a key role in development and is essential in the removal of cells during the metamorphosis of insect and amphibia. Typical morphological features characterize cells undergoing apoptosis that allow apoptosis to be morphologically different from necrosis.

This cell death pattern usually concerns scattered cells within healthy tissue that are selectively removed by phagocytosis by neighboring cells. The active phagocytosis of apoptotic cells plays a major role in preventing the inflammatory reaction which could derive from their uncontrolled degradation. However, the molecular mechanisms allowing recognition and ingestion by bystander cells of apoptotic cells are not yet entirely understood. It has been demonstrated that during the onset of the apoptosis, several immature glycans are exposed on the surface of apoptotic cells. A relevant presence of galactose and mannose residues on the cell surfaces of apoptotic bodies and the ability of the specific receptor antibodies and sugar moieties to block their binding and uptake by the living liver cells (i.e. hepatocytes, Kupffer and endothelial cells) support the conjecture that the galactose-specific receptor is involved in the clearance of apoptotic cells.

In the present study, to verify whether also the mannose-specific receptors of liver cells are involved in the phagocytic process of apoptotic hepatocytes and circulating apoptotic cells we investigated the cell surface receptor expression of mannose specific receptors during the process of apoptosis induced by the i.v. injection of lead nitrate in rats. We measured an independent modulation of the cell receptor expression on the three liver cell types. Hepatocytes show a drastic decrease of binding sites soon after the beginning of the treatment that coincides with the phase of cell proliferation. A large increase of receptors is observed at day 15 after the lead nitrate injection. A similar pattern was observed on endothelial cells with the exception of an increase of receptors around the 7th day from the injection. Kupffer cells present two phases of increased receptor expression: one at day 1 from the beginning of treatment and a second and more relevant increase at day 10 after the lead nitrate injection.

We-170

FASTING INDUCES APOPTOSIS OF PRENEOPLASTIC CELLS AND IMPAIRS THE RATE OF HEPATO-CARCINOGENESIS

B. Grasl-Kraupp, B. Ruttkay-Nedecky,
A. Wagner, W. Bursch and R. Schulte-Hermann

Institut für Tumorbioologie-Krebsforschung, Borschkegasse 8a, A-1090 Wien, Austria

Restriction of dietary calories reduces cancer formation in all species tested so far. We studied the effect of fasting on physiological cell death through apoptosis using rat liver as a model. In normal liver involution of hyperplasia by apoptosis was reinforced by food withdrawal and suppressed by feeding. Complete food withdrawal for 8 days or food reduction by 40% for 3 months eliminated 20-30% of normal liver cells through apoptosis. Putative preneoplastic liver foci (PPF) exhibited severalfold higher rates of DNA replication and apoptosis than unaltered liver. Food restriction lowered DNA replication but increased apoptosis, which reduced number and volume of PPF by 85% within 3 months. Subsequent return to ad libitum feeding normalized cell replication and apoptosis but clear differences in volume and number of PPF persisted during the following 17 months. Treatment with nafenopin, a peroxisome proliferator and potent tumor promoter, produced only half as many hepatocellular adenomas and carcinomas as in animals fed always unrestricted. This implicates that by induction of apoptosis food restriction had actually extinguished part of the initiated cells. This effect may provide protection from cancer.

We-171

REGULATION OF APOPTOSIS AND PROLIFERATION BY PROTEIN PHOSPHORYLATION IN B-LYMPHOMAS

R. Mihalik¹, L. Kopper², M. Benczúr¹

¹ Dept. of Immunol., Natl. Inst. of Hematol. and ² Inst. of Pathol. and Exp. Canc. Res. Semmelweis Univ. Med. School, Budapest, Hungary.

Protein phosphorylation/dephosphorylation takes part in the biochemical pathways regulating the cell proliferation and apoptosis. We have investigated the modulation of proliferation and cell death of a human non-Hodgkin lymphoma cell line of B-cell origin (HT-58) by phorbol ester PMA and protein kinase inhibitor staurosporine (STA).

Low concentration (conc.) of PMA (0.5 ng/ml) ceased proliferation after 8h. in G1 phase for a prolonged time. Higher conc. PMA (>5 ng/ml) first delayed cell cycle in G2 till 4h. followed by arrest in G1. The concomitant induced cell death was low on the first day of treatment, and increased up to 50% on the third day, detected by flow cytometry.

STA at 25 nM partially delayed proliferation in G1, while higher conc. (>50 nM) arrested cells in G2 with concomitant, conc. dependent cytotoxicity in the form of apoptosis, which was mainly cell cycle independent.

However, a short pretreatment (>2h.) with PMA (>0.1 ng/ml) sensitised the lymphoma cells for apoptosis induced by low conc. STA. Synergistic effect reached plateau phase by 12h. cotreatment, and was also cell cycle independent.

These data indicate a complex regulation of proliferation and apoptosis by protein phosphorylation, and propose a pharmacological model for studying apoptosis in lymphoma cells.

Genes involved in neuronal survival

We-172 Jean-Claude Martinou and Rémy Sadoul
Glaxo Institute for Molecular Biology, 14 chemin des Aux,
Plan les Ouates, CH-1228, Geneva, Switzerland

In an attempt to dissect the molecular mechanisms controlling neuronal cell-death, we have developed a model system based on the strict requirement of sympathetic neurons for NGF. These neurons die by apoptosis soon after NGF has been removed from the culture medium. Expression vectors microinjected in these neurons allows us to test for effects of different proteins on neuronal survival. Using this model, we and others have shown that neurons can be rescued by the proto-oncogene Bcl-2. We now demonstrate that overexpression of Bcl-XL, a protein homologous to Bcl-2 and strongly expressed in adult brain, is also able to block neuronal cell-death. Bcl-Xs, a splice-variant of Bcl-X lacking an internal domain of 68 amino-acids, has no protective activity but is able to counteract Bcl-XL activity. These results suggest that alternative splicing of genes encoding Bcl-2 homologues may be a means for regulation of neuronal cell death and thereby for neuronal selection. To test this we have made transgenic animals which overexpress Bcl-2 before and during the developmental period of normal neuronal cell death. We found that the retina and facial nucleus of these animals show a 30 to 40 % excess of neurons which persists until adult age. This demonstrates that some, but not all of the neuronal selection which occurs during development uses pathways which may be blocked by Bcl-2. Our results suggest a regulating role for bcl-2 related genes such as bcl-x in neuronal selection. Overexpression of bcl-2 in transgenic animals was also shown to protect facial motoneurons from axotomy and cortical neurons from ischemia induced by permanent occlusion of middle cerebral artery. Therefore we believe that Bcl-2 and related proteins may represent novel therapeutical targets for treatment of acute and chronic neurodegeneration.

Another gene also able to rescue neurons in vitro is the tumor suppressor P53. Overexpression of P53 has been shown by others to block cells in the G1 phase of the cell cycle. The protective role of P53 on postmitotic neurons that we observed may therefore highlight a relationship which could exist between neuronal cell-death and mitosis.

**MEMBRANE PERMEABILITY AS A CRITERION
TO EVALUATE APOPTOTIC CELLS INDE-
We-173 PENDENTLY OF DNA BREAKS**

L.Zamai^a, E.Falcieri^{bc}, S.Santi^c, C.Cinti^d, P.Gobbi^a
and M.Vitale^c

^a Inst. Anatomia Umana Normale, Univ. Bologna; ^b Inst. Morfologia Umana Normale, Univ. Chieti; ^c Inst Citomorfologia N.P. CNR, c/o Inst. Codivilla-Putti, Bologna; ^d Lab. Biologia Cellulare e Microscopia Elettronica, IOR, Bologna; ^e Dept. Scienze Biomediche e Biotecnologie, Anatomia Umana, Univ. Brescia, Italy

One of the main limitations to apoptosis detection in flow cytometry is the impossibility to recognize apoptotic cells when DNA cleavage does not occur. The propidium iodide (PI) uptake in apoptotic cells, due to an increased membrane permeability, allows to overcome the problem. This technique can substitute DNA staining of ethanol-treated cells or nick translation, that need DNA cleavage, to recognize apoptotic cells. Molt-4 human leukemia cells treated with staurosporine were used as source of apoptotic cells without DNA breaks and were compared with camptothecin-treated HL-60 human leukemia cells, in which DNA cleavage can be identified by DNA gel electrophoresis. The subdiploid peak, which identifies the apoptotic HL-60 ethanol-treated cells by flow cytometry, does not appear in Molt-4 cell apoptotic model, despite the presence of apoptotic ultrastructural features (Falcieri et al. Biochem Biophys Res Comm 193,19,1993). Nevertheless, when PI uptake is studied in fresh cells, a progressive incorporation can be demonstrated in both apoptotic models, which is not observed in healthy cells (Vitale et al. Histochemistry 100,223,1993). The PI incorporation in fresh apoptotic cells, closely correlated to the membrane permeabilization, can so be considered a valid approach to identify apoptotic cell population by flow cytometry.

**CHANGES IN THE PROGRAMMED CELL DEATH
OF POLYMORPHONUCLEAR GRANULOCYTES
WITH AGING UNDER VARIOUS STIMULATIONS**

We-175 T. Fülop Jr^a, C. Leblanc^a, J. Seres^b, L.Bene^c and S. Damjanovich^c ^aDept. de Médecine et Centre de Rech. en Gérontol. Gériat, CHUS et Hop. d'Youville, Sherbrooke, Qc, Canada, ^b1st Dept. of Medicine, and ^cDept. of Biophysics Univ. Med. Sch. of Debrecen, Hungary,

It is well known that apoptosis is playing an important role in several diseases such as cancer. In aging many alterations are occurring at cellular and organic levels. Our aim was to study the apoptosis of polymorphonuclear granulocytes (PMNLs), as a model, in aging under several stimulations, as the apoptosis might play a role in several pathologies encountered with aging. The PMNLs of healthy young (20-25yrs), middle aged (35-50yrs) and elderly (65-yrs) were studied for apoptosis after 18 hours of sterile culture without or with stimulation. The agents used were: PMA, H₂O₂, G-CSF, GM-CSF, reduced glutathione, glutathione peroxidase. The apoptosis was assessed by traditional staining of the plates and by flow cytometric staining. It was found that without stimulation the susceptibility of PMNLs to apoptosis was decreased with aging. Under various stimulations such as PMA, H₂O₂ was almost 100%, while the other stimulants could prevent to different extent the apoptosis. Marked age-related changes were observed in the extent of apoptosis under stimulation with these agents. The antioxidant agents were able to prevent to some extent the apoptosis mainly in young subjects. We think that the changes in apoptosis with aging could play a role in certain pathologies of aging, such as cancer, Alzheimer's disease.

**Intracellular vesicles (IGV's) of cytotoxic T-
lymphocytes: their role in apoptotic target cell death**

We-174 Schaefer, E., O.Karapetian, M.Adrian and J.Tschopp,
Institute of Biochemistry, University of Lausanne, 1066
Epalinges

Appropriately stimulated cytotoxic T-cells (CTL) accumulate storage compartments, granules, as multivesicular bodies that eject their contents in a regulated exocytic response to induce the apoptotic cell death of the target cell. The granules harbour the lytic pore forming protein perforin, serineproteases (granzymes) that have been described to play a role in the target cell (TC) attack. The cascade of events driving the TC to death however is not yet unravelled.

We now show that the granule proteins are released in a heterogenous population of membrane surrounded vesicles of 80-110 nm diameter, visualized by electronmicroscopy. These intracellular vesicles IGV's contain granule specific proteins as perforin, granzymes, calreticulin as well as T-cell receptor.

Are these vesicles the vehicles for CTL proteins to be injected into the target cell for its destruction? Incubation of IGV's with sensitized but not with unsensitized TC results in the degradation of the TC-DNA, suggesting a specific fusion of the IGV with the TC plasma membrane. Autoradiogram of electronmicrographs revealed association of IGV specific proteins with the nucleus already 15 minutes after incubation of IGV with target cells. In the nuclear fraction of target cells, 3 IGV specific proteins are associated. One of the protein has been identified as granzyme A. We propose that CTL specific proteins are transferred to the TC via vesicles that interact specifically with the target cell, and are translocated to the TC nucleus to induce the lethal hit.

**EXTRACELLULAR DNA IN THE SERA OF
PREGNANT WOMEN**

We-176 V.Kazakov, V.Bozkov, V.Linde and
V.Mikhailov

Institute of Cytology RAS, St.Petersburg, Russia

Morphological study of decidua cells demonstrated the gradual DNA loss by nuclea at terminal stage of differentiation. It was decided to be manifestation of programmed death of decidua cells. It was proposed that some products of apoptotical cells may be observed as sera DNA. The analyses of seras revealed the increased amount of low molecular weight DNA in seras at ending of pregnancy as compared with the seras of men, nonpregnant women and women at beginning of pregnancy. The size of such DNA is between 150 and 2500 bp. The PCR technique allowed us to detect sera DNA ALU and inter-ALU sequences at the beginning of pregnancy and ALU sequence at the ending of pregnancy. The size of inter-ALU stretches is up 1 kb.

We-177 MODIFICATIONS IN ENZYMATIC ACTIVITIES AND IN MEMBRANE STRUCTURE DURING ERYTHROCYTE AGING IN DIABETES MELLITUS
 R.A. Rabini, R. Staffolani*, E. Salvolini**,
 G. Biagini**, P. Fumelli, L. Mazzanti*
 INRCA Hospital, Ancona,*Institute of Biochemistry and **
 Institute of Human Morphology, University of Ancona (ITALY)

Insulin-dependent diabetes mellitus produces an accelerated aging process in the plasma membrane. We fractionated erythrocytes (RBC) from 18 normal subjects and 21 IDDM patients in 5 subpopulations of different mean age by Percoll BSA density gradients as described by Salvo. Plasma membranes were prepared by the method of Burton; Na+/K+ ATPase and acetylcholinesterase (AchE) activities were determined respectively as described by Kitao and Hattori and by Ellman. The protein structural pattern within the membrane was studied by the freeze-fracturing technique. Na+/K+ ATPase activity decreased during aging and was higher in all RBC subpopulations from normal subjects than in IDDM. AchE decreased during aging in normal subjects, while it increased in IDDM. The structural study showed increased diameter of intramembrane particles in IDDM in all the fractions studied. We hypothesize that altered enzymatic activities during RBC aging in IDDM might be related to modified interactions of protein subunits within the membrane.

We-179 EFFECTOR ELEMENTS IN THE MOLECULAR PROGRAM OF NATURAL CELL DEATH FORMS
 Laszlo Fesus
 Department of Biochemistry, University Medical School, Debrecen, Hungary H-4012

Intense cellular and molecular studies of recent years have revealed the existence of evolutionary conserved but variably appearing forms (programmed cell death, apoptosis, terminal differentiation) of active cell death. The biochemical entity of the killer protein(s) is not known. Part of the gene products which are induced and/or activated during the death process are effector enzymes with known function but unclear roles in death: DNA degrading enzymes (DNase I, DNase II, endonucleases), proteases (particularly the interleukin-I converting enzyme, ICE) and protein cross-linker transglutaminases. The latter Ca-dependent enzymes form high molecular weight protein polymers in almost all types of cells dying in either of the three forms of natural death. Overexpression of the most often involved tissue transglutaminase by transfection leads to, similarly to DNase I or ICE, increased rate of cell death. Inhibition of transglutaminase expression or activity results in either prevention of cell death or a death form resembling necrosis with uncontrolled disintegration. It is suggested that effector elements of natural cell death can function both as killer enzymes and as protective molecules palliating symptoms of individual cell death for the benefit of the whole organism.

We-178 APOPTOSIS IN TARGET ORGANS OF HYPERTENSION
 Hameet P, Richard L, Dam TV, Teiger E, Gossard F and Tremblay J, Centre de recherche, Hôtel-Dieu de Montréal, Université de Montréal, Montréal, Canada.

Introduction: Disorders in the physiology of vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) include an increased growth rate, a decrease in the length of the cell cycle and an increased sensitivity to growth factors. The increased DNA synthesis, present already in neonates from genetically hypertensive parents is not always accompanied by a higher DNA content, suggesting an acceleration of the cellular turnover.

Methods: Different methods were used to detect programmed cell death (apoptosis) in VSMC and organs of control and hypertensive animals: a) Morphological alterations of apoptotic cells. b) Presence of approximately 200bp ladder DNA fragments on agarose gel. c) In situ detection of DNA breaks by terminal deoxynucleotidyl transferase assay.

Results: All three methods used demonstrated an increase of apoptosis in VSMC, heart ventricles, medulla and in a lesser extent the corticomedullar region of the kidney from SHR and hypertensive mice. Apoptosis was increased by plasmin (via maturation of TGF β) and this increase in SHR is overcome by the addition of TNF.

Conclusion: This study shows, for the first time, an increased apoptosis in target organs of hypertension. This concept of increased growth as well as of apoptosis opens new understanding of pathophysiology and treatment opportunity in cardiovascular disease.

We-180 TRANSGLUTAMINASE AND INVOLUCRIN EXPRESSION DURING APOPTOSIS OF CHO CELLS OVEREXPRESSING c-MYC
 Z. Balajthy, E. Susan, and L. Fesus
 Department of Biochemistry, University Medical School, Debrecen, Hungary H-4012

Several of the molecular elements of apoptosis, a cell death process originally described by morphologic criteria, have been recently described. One of these elements is the protein cross-linker, Ca²⁺-dependent tissue transglutaminase^{Fesus et al. Eur. J. Cell Biol. 56, 170, 1991}, which is induced and activated during apoptosis. However, it is not known what regulates this enzyme and its substrate during the death processes. Heat shock-inducible overexpression of c-Myc in Chinese hamster ovary (CHO) cells^{Bissonnette et al. Nature. 359, 552 1992} results in cell death.

We have found that apoptosis is induced by heat or heat plus c-Myc overexpression in these cells when they are in the mitotic cycle; however this doesn't occur in the confluent state. The level of transglutaminase protein and its activity is not changed upon induction of apoptosis, but its product, the protein-bound ϵ - γ -glutamyl/lysine cross-link is significantly increased. In further experiment β -galactosidase reporter gene constructs containing 5' regions of the transglutaminase and involucrin have been used in transient transfection assays to clarify how these genes are regulated during the cell death process.

INCREASED APOPTOSIS IN PSORIASIS

We-181 E. Remenyik ^a, V. Thomazy ^b, P.J.A. Davies ^c,
M. Duvic ^d

^{a, d} Dept. of Derm., ^d Int Med and ^{b, c} Pharm, Univ Tx Medical Sch, Houston, TX , USA and ^a Dept. of Derm Med. Sch Debrecen, Hungary

Normal epidermal differentiation is characterized by keratinocyte programmed cell death (specialized apoptosis) which occurs in the upper layers, the stratum granulosum and corneum. Psoriasis is a disorder characterized by a TH1 immune reaction which is accompanied by abnormal epidermal differentiation and a thickened stratum corneum. In order to determine whether apoptosis was altered in psoriasis we studied skin biopsies taken from uninvolved and lesional skin from 5 psoriatic patients using the TUNEL method which detects fragmented DNA, characteristic of apoptotic cells. Formalin fixed paraffin sections were digested with proteinase K for 15', incubated with terminal transferase and biotinylated dUTP conjugates (Boehringer-Mannheim), and detected with streptavidin-biotin with either DAB or alkaline phosphatase (DAKO) immunohistochemistry. Keratinocyte transglutaminase (TGase I) and involucrin were also detected by immunoperoxidase. Staining of cells undergoing nuclear fragmentation was detected in the abnormal parakeratotic cell layer of psoriasis lesions and in the stratum granulosum of uninvolved psoriatic skin. There were increased numbers of apoptotic cells in psoriasis compared with normal. Apoptotic nuclei were present in cells expressing differentiation markers, TGase I and involucrin. Increased epidermal apoptosis may be involved in the pathogenesis of psoriasis and may be mediated in part by the action of TGase I.

CHANGES IN THE IN VITRO AND IN VIVO

TRANSGLUTAMINASE ACTIVITIES OF MOUSE
We-182 THYMOCYTES DURING APOPTOSIS INDUCED VIA
p53 DEPENDENT AND INDEPENDENT SIGNAL
TRANSDUCTION PATHWAYS

Z. Szondy, M. Boyadjisz, A. Mádi, E. Susán and L. Fésüs

Department of Biochemistry, University Medical School of Debrecen, Debrecen, Hungary

Apoptosis is a programmed mechanism of cell death. In thymus it functions to prevent autoimmunity and to dispose T cells expressing low affinity receptors. Despite of the intensive studies of the last 10 years the biochemistry of the apoptosis is not well known. Previous work in our department has suggested that in some of its forms the tissue transglutaminase may be activated. Apoptosis of thymocytes can be induced via 3 different signalling pathways: 1. via the T cell receptor requiring the function of myc and nur77; 2. by addition of dexamethasone; 3. by addition of etoposide (a topoisomerase inhibitor), or by high dose of irradiation. The last 2 forms belonging to point 3. require the presence of p53, a tumor suppressor, in the signalling pathway. In our studies it was investigated both in an *in vitro* and an *in vivo* system whether transglutaminase activation is related to any of these forms of cell death. In the *in vitro* system we could not detect any change in the transglutaminase activity though the concentration of its crosslinked product increased with time. In the *in vivo* system transglutaminase activity and protein level significantly increased after apoptosis induction in both the p53 dependent and independent systems. Further examinations are in progress to study whether the observed *in vivo* transglutaminase activation is related to specific environmental effects on thymocytes or to tissue rearrangement concomitant of thymocyte loss.

MORPHOLOGICAL AND BIOCHEMICAL
FEATURES OF SUNBURN CELL

We-183 FORMATION OF CULTURED HUMAN
KERATINOCYTES

N. Wikonkál, É. Remenyik, Á. Kósa, L. Fésüs*, I. Horkay
Dept. of Dermatology, *Dept. of Biochemistry, University Medical School of Debrecen, Hungary

Sunburn cells (SBC) with their specific morphological appearance can be seen after exposure of normal human epidermis to UV radiation. The microscopic picture of these cells is quite distinct, similar to those cells which are undergoing the process of apoptosis. This similarity is also supported by the relatively fast appearance and rapid elimination of SBCs. The role of SBCs is not completely understood, either in healthy persons or in patients with light sensitivity skin disorders. Our goal is to study the exact time and dose dependence of the SBC formation on cultured human keratinocytes. Cultured keratinocytes were irradiated with different wavelengths of UV light by a xenon-arc solar simulator. We examined the dose dependency and the optimal latency period of SBC formation and biochemical markers of this process. Sunburn cell formation of keratinocytes obtained from healthy and light-sensitive patients was compared.

APOPTOSIS (PROGRAMMED DEATH) IN
We-184 COLONIC CELLS.

S. Lifshitz^a, B. Schwartz^a, S. Polak-Charcon^b,
R. Mikhailowski^a, Y. Niv^a, S.A. Lamprecht^a.

^aGastroenterology Department and Clinical Biochemistry Unit, Soroka Medical Center, Ben-Gurion University of the Negev, Beer-Sheva; ^bInstitute of Pathology, Sheba Hospital, Ramat-Gan, Israel.

The orderly progression of tissue growth and differentiation requires the death and removal of individual cells without disruption of the society from which they originate. This form of altruistic programmed cell death is known as apoptosis (programmed cell death). We propose that apoptosis plays a central role in the maintenance of colonic cell number and the ordered renewal of colonic cells. Apoptotic-related events were studied in rat colonocytes harvested from the crypt continuum at precise stages of their biological life span. Degradation of DNA into oligonucleosome-sized fragments, a hallmark of apoptosis, was measured in the murine colonocytes. The characteristic nucleosome ladder was detected by gel electrophoresis in rat colonocytes harvested at all stages of proliferation/differentiation. Apoptosis was confirmed by the *in situ* end-labelling of DNA strand breaks. Ultrastructural correlates provided typical morphological evidence for the programmed demise of the colonic cell. The activity of transglutaminase, an enzyme which catalyzes the cross-linking of proteins, was found to be predominant in differentiating and mature colonocytes. Of note, colonocytes collected in clusters - presumably maintaining intercellular communications - retained a normal structural organization. A key question addressed pertains to the cellular signals which control cell survival and cell death in a precise zonal and temporal context within the colonic crypt. The present findings support the notion that the apoptotic process is part of the stringent regulatory mechanisms that keep constant the boundaries of the colonic compartments.

We-185 STARVATION-INDUCED PROGRAMMED DEATH OF HYBRIDOMA CELLS: PREVENTION BY NEUTRAL AMINO ACIDS

E. Franček and K. Chládková

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Praha 4, Czech Republic

Murine hybridomas were found to die by apoptosis due to a variety of stimuli, including deviations from optimum culture conditions. Cells transferred into medium diluted to 50% or less by saline displayed characteristic features of apoptosis.

The starvation-induced death could be prevented by certain additives to the diluted medium. Amino acid mixtures (MEM essential amino acids, MEM non-essential amino acids) were active in death prevention when applied at 1-2 mM concentrations, while addition of vitamins was quite ineffective.

Testing of the effect of individual amino acids revealed that the starvation death could be prevented by alanine, serine, glycine, proline, threonine, asparagine, glutamine, and also β -alanine. The same neutral amino acids with unbranched side-chains have been known to act as repressors of the adaptively regulated amino acid transport system A.

The finding indicates that extracellular levels of certain amino acids may control not only the up- and down-regulation of transmembrane transport but also the triggering of apoptotic death. It is suggested that these amino acids act under certain circumstances as repressors, or co-repressors, of the switch from mitosis to apoptosis.

Supported by the Grant Agency of the Czech Republic (No. 503/93/2292).

We-186 CELL DEATH IN VITRO - DYNAMICS OF STRUCTURAL CHANGES AFTER TREATMENT WITH XENOBIOTICS

M. Červinka, V. Půža

Department of Biology, Charles University, Medical Faculty, Hradec Králové, Czech Republic

The purpose of this study was the analysis of structural changes connected to in vitro cell death induced by several model xenobiotics. The main emphasis was given to the detailed analysis of the dynamic of these changes.

Our experimental model of cell death include both apoptosis (cisplatin) as well as necrosis (Tween20) in stabilized cell lines (Hep2 - human epithelial cells and L929 - mouse fibroblast). The dynamic of cellular changes was followed by time-lapse microcinematography. Quantification of the data (cell volume, cell shape, the nucleus shape and size) was done by computer image analysis system (LUCIA-M).

In our experiments we try to exactly define cell death on the basis of laborious analysis of the fate of single cells before, during and after the treatment with model drugs.

By the analysis of the dynamic of structural changes leading to the cell death we were not able to find relevant, single, non-ubiquitous marker of cell death. Nevertheless we believe that our data bring new information to the discussion about possible intermediate (between apoptosis and necrosis) types of cell death. Our data help to develop general model of cell death. This is basic scientific background for the search of new in vitro cytotoxicological tests.

G PROTEINS INVOLVED IN THE CALCIUM CHANNEL SIGNALLING

We-187 J. Hescheler, F. Kalkbrenner, V.E. Degtyar, B. Wittig* and G. Schultz
Institut für Pharmakologie (Thielallee 69/73), *Institut für Molekularbiologie und Biochemie (Amimallee 22), Freie Universität Berlin, D-14185 Berlin, FRG

In order to identify the heterotrimeric G proteins involved in the inhibition of Ca channels by various receptors, we injected anti-sense oligonucleotides hybridizing to the mRNAs encoding the α , β and γ subunits of G proteins into the nuclei of pituitary cells of the line GH₃. Using this system, we previously demonstrated the involvement of $\alpha_0\beta_3\gamma_4$ and $\alpha_0\beta_1\gamma_3$ in the inhibition of L-type Ca channels by acetylcholine and somatostatin, respectively. In order to investigate whether the same triplets may be involved in inhibitory effects by other receptors we determined the G proteins coupling galanin receptors to L-type Ca channels. After injection of $\alpha_0\gamma_1$ antisense oligonucleotides, inhibition of Ca currents was reduced from 20% in control injected cells to 5% in anti- $\alpha_0\gamma_1$ injected cells. Injection of β_2 and β_3 antisense oligonucleotides decrease the Ca current inhibition to 10% and 15%, respectively. Injection of γ_2 and γ_4 antisense oligonucleotides decreased it to 10% and 12%, respectively. Corresponding experiments in pancreatic B-cells of the line RINm5FT revealed similar combinations of antisense effects. Our data suggest that the inhibition of L-type Ca channel by galanin is mediated by two heterotrimeric G proteins, mainly by $\alpha_0\beta_2\gamma_2$ and less efficiently by $\alpha_0\beta_3\gamma_4$, a combination which is also used by muscarinic M₄ receptors in these cell lines.

CANCELLED

PERTURBED CELL SIGNALLING AFFECTS FACTORS
CONTROLLING CNS GROWTH DURING CHICK
NEUROGENESIS

We-189 ^aC. Angelini, ^aC. Falugi, ^bG. Cimoli

^aInstitute of Comparative Anatomy, University, Genova, Italy;
^bNational Institute for Cancer Research, Genova, Italy

The regulation of intercellular signalling is crucial for correct development, during the early inductive interaction that specifies the vertebrate body plan. In particular, neurogenesis seems to be led by cholinergic molecules (Filogamo and Marchisio, *Neurosci. Res.*, 4, 29, 1971) and the acetylcholine (ACh) level was found to correspond to NGF presence in the vertebrate CNS (Thoenen et al., Horwitz Series, 1987, p.379).

ACh receptors-active drugs were tested on chick early development and their effect was correlated to the expression of cholinergic molecules, NGF and to the intracellular distribution of protein kinases by histochemical and immunohistochemical methods. *Atropine*, rather selective antagonist of the G-protein-linked ACh muscarinic receptor; *carbamylcholine*, agonist of the same receptor; *nicotine*, agonist of Na⁺ channel-gating Ach receptor, and *acetylcholine* were applied at different dilutions in chick Tyrode solution, in developing embryos: a) stage 5 = gastrulation and cephalic process; b) stage 10-11 = trunk and caudal neurulation. Total incubation lasted 5 days.

The drugs effect depended both on the drugs concentration and on the stage when they were applied. At higher concentration the effect was always inhibitory of development and often lethal. At the lower concentration, the effect was inhibitory for *atropine*, that enhanced the expression of muscarinic-like molecules in the central nervous system, but decreased the NGF distribution pattern; on the other hand, *carbamylcholine* enhanced development, if applied at stage 10-11, and inhibited it, when applied at stage 5. *Carbamylcholine*-treated embryos showed decreased muscarinic-like molecules expression, and a strongly increased NGF-like immunoreactivity distribution in the CNS, including ependimal channel. *Nicotine*, at every dilution, caused strong anomalies, e.g. external heart, death of embryos, etc.

PIG DEVELOPMENT OF NATURAL KILLER
CYTOTOXICITY WITH MODIFIED C-6 GLIOMA
CELL LINE AS TARGETS

We-191 H.Kovářů ab, F.Kovářů bc, R.Halouzka c, and
H.Kozáková b

a 1st Med. Fac., Charles Univ., Prague, b Inst. Microbiol.,
Acad. Sci., Prague, c Univ. Veter. Sci. Pharm., Brno, Czech
Rep.

It has been suggested that the pigs display negligible natural killer (NK) activity of blood lymphocytes at birth followed by slight development during first weeks (1,2) with human erythroleukemic K 562 cells as targets.

Our aim was to analyze a possibility to prove NK cytotoxicity, as one of basic surveillance mechanisms in the organism, during fetal and early postnatal pig lymphocyte development.

We used new target cells modified by us for NK activity assays, i.e. rat astrocytoma C-6 cells with inserted pig thymocyte cell surface determinants. These targets represent combination of xenogeneic and syngeneic determinants of cell surface for recognition and cytolytic NK reactivity of tested cells. On fetal day 100, we demonstrated significant NK cytotoxicity of blood lymphocytes (25% of specif. ⁵¹Cr release) comparing to background values with K 562 or original C-6 cells. Cytotoxic activity was developed markedly on day 35, because of higher values with modified C-6 glioma targets (56%) comparing to 22% with K 562 targets and 4% with original C-6 glioma cells.

1. Huh N.D., Kim Y.B., Koren N.S., Amos D.B.: *Int. J. Cancer*, 1981, 28: 175. 2. Kim Y.B., Ichimura O.: In *Swine in biomedical research*. Ed.M.E.Tubleson, Plenum Publ. Corp., Vol.3, 1986: 1811.

Detection of 28 and 65KDa proteins in
Mycobacterium avium intracellulare Com
plex(MAC) infected human macrophages.

We-190

Flora Ippoliti, Dep.of Exp.Med."La Sa-
pienza" University.Rome - Italy.

Mycobacterium avium intracellulare complex (MAC) is the most common bacterial pathogen isolated from AIDS patients. To investigate if the members of the heat shock proteins released by mycobacteria during growth are available for immune recognition we monitored the presence of both 28 and 65 KDa proteins in a model of CD68+ human macrophages infected with MAC transparent colonial variant (serovar 21 from AIDS patient) and cultured for 10 days. To this end H60 and H105 monoclonal antibodies recognizing M.tuberculosis 28 and 65 KDa proteins cross-reactive with MAC in ELISA test, were used in an immunocytochemical assay.

MAC efficiently proliferated within cells. Macrophage positivity for 65 and 28 KDa antigens was 3,48,64,50% and 0,15,21,48% respectively on days 0,5,7,10, suggesting a modulated expression of the two antigens during in vitro infection. Uninfected or LPS-treated macrophages were negative.

These result is according to the lack of induction of IL-1, IL-6, TNF_α, GM-CSF and G-CSF from infected macrophages by MAC transparent colonial variant.

IMMUNOPHENOTYPIC CHARACTERIZATION
OF PIG LYMPHOCYTES IN PRENATAL

We-192 DEVELOPMENT

F.Kovářů, H.Kovářů a, R.Halouzka,
I.Zendulká, and J.Drábek

Univ. Veter. Sci. Pharm., Brno, a 1st Medical Fac., Charles
Univ., Prague, Czech Rep.

We assayed quantitative proportions of fetal pig T lymphocyte subpopulations (CD4, CD8 and CD2) using flow cytometry (FACS) and immunofluorescent microscopy with monoclonal antibodies and FITC or PE conjugates with second antibody.

Thymus. Subpopulations of CD4 and CD8 lymphocytes were demonstrated very early on day 51. We can suggest from interpolated developmental curves first occurrence of both subpopulations around day 40, when thymus is not still differentiated into cortex and medulla.

Spleen. On day 51, CD2 lymphocytes were detected only, whereas CD4 and CD8 cells were demonstrated on day 60. Number of CD4 and CD8 lymphocytes was multiplied during subsequent development up to 3 orders per organ.

Lymph nodes. We tested day 90 only. Proportions of both CD4 and CD8 lymphocytes were higher comparing to spleen ones.

CD4 / CD8 ration. In thymus and spleen is value near to one at birth with slightly dominant CD4 lymphocytes. In lymph nodes, CD4 / CD8 index = 0.85 at birth, as estimated recently for adult pig lymphocyte subpopulations.

We-193

MHC CLASS II-RESTRICTED ANTIGEN
PRESENTATION IN TRANSGENIC MICE
EXPRESSING A RAB5 INHIBITORY MUTANT
PROTEIN
J. Boretto, P. Ferrier, P. Naquet, J.P. Gorvel and
P. Chavrier

Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9 France.

Recent *in vivo* and *in vitro* studies have shown that ras-related GTPases belonging to the rab subfamily, play a role in controlling membrane traffic. The rab5 protein is associated to the cytoplasmic face of the plasma membrane and early endosomes. *In vitro*, rab5 is required for the homotypic fusion of early endosomes. Overexpression of the rab5^{Ile133} mutant protein *in vivo* decreases the rate of endocytosis and leads to the appearance of abnormal small tubules and vesicles at the periphery of the cells. Class II major Histocompatibility molecules (MHC II) bind antigenic peptide fragments of endocytosed antigens for presentation to helper T cells. Antigenic peptides production and association with MHC II molecules are likely to take place in the degradative endosome-lysosome pathway.

In order to directly address the function of rab5-controlled endocytosis in antigen presentation we have generated transgenic mice expressing the mutant rab5^{Ile133} protein in their B lymphocyte presenting cells. The sequence encoding rab5^{Ile133} (N-terminally tagged with the N-myc epitope) has been inserted in a vector allowing its transcription from promoter/enhancer elements of the IgH gene locus. Transgenic lines have been derived from three independent founders by crosses with CBA/J mice. The transgenic messenger is strongly expressed in thymus, spleen and bone marrow (not detected in brain, kidney and liver). The mycRab5^{Ile133} protein could be immunoprecipitated from metabolically labeled splenic and thymic cells. Our efforts are now focused on a detailed analysis of the surface markers expressed by the B and T cell compartments of the transgenic animals by flow cytometry. In parallel, we will determine whether the expression of the rab5 mutant protein interferes with the endocytic and antigen presentation properties of splenic B cells *in vitro*, as well as antibody response in the transgenic mice.

We-195

DISTRIBUTION OF THE GOOSE PARVOVIRUS
ANTIGEN IN INFECTED EMBRYONAL GOOSE
CELLS

M.Alexandrov^a, L.Doumanova^a

^a Bulgarian Academy of Sciences, Institute of General and Comparative Pathology; ^b Central Research Veterinary Institute, Department of Virology, 1606 Sofia, Bulgaria.

Immunocytochemical investigations were carried out to establish the goose parvovirus antigen(GPVA) in infected embryonal goose cells (EGC). Direct immunofluorescent and direct immunoperoxidase techniques were used.

Examinations were performed at 12-18-24-36-48-72 and 96 hours post infection.(p.i.).

It was found that the GPVA appeared first in the nuclei at 18.h.p.i. as small irregular distributed granules which later arised. At the 24. h.p.i. nuclei with slight homogenous staining for GPVA also were appeared.

After the 72.h.p.i. homogeneous stained nuclei were predominated.

At the same time cells with cytoplasmic distribution of the antigen as well as well definitive cytopathic effect among cells monolayer were also observed.

We-194

IN THYMIC EPITHELIAL CELLS OF TAP1 KNOCK-OUT MICE MHC CLASS I MOLECULES ACCUMULATE IN A PRE-GOLGI RETICULUM ENRICHED IN UBIQUITIN AND UBIQUITINYLATION ENZYMES

G. Raposo^a, H.M. van Santen^b, R.L. Leijendekker^a, H. Ploegh^b, and H.J. Geuze^a.

Department of Cell Biology, Utrecht University, AZU H02.314, 3584 CX Utrecht, The Netherlands^a and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Mass. 02139, U.S.A.^b.

Class I Major Histocompatibility Complex (MHC) molecules bind peptides derived from the degradation of self and foreign antigens respectively, and display them on the surface of antigen presenting cells (APCs) for recognition of the MHC-peptide complexes by T-lymphocytes. Class I bound peptides are generated in the cytosol and are translocated into the lumen of the endoplasmic reticulum (ER) by the transporters associated with antigen presentation, TAP1 and TAP2. In order to analyse the importance of bound peptides in the intracellular transport of newly synthesized human and mouse class I molecules, mice that are both transgenic for HLA-B27 and knock-out for TAP1 have been produced. Using immunocytochemistry at the light- and electron microscopical level, we have analysed the localisation of HLA-B27 in thymic tissue from the transgenic mice either in a normal or in a TAP1 deficient background. HLA-B27 as the endogenous mouse class I is expressed at high levels in thymic epithelial cells. In these cells class I molecules are localized over the plasma membrane and in the compartments of the biosynthetic pathway. In thymic epithelial cells deficient in TAP1, class I molecules are absent from the plasma membrane and are massively retained in an extremely extended cis-Golgi reticulum or ER-Golgi intermediate compartment. The intermediate compartments are very likely involved in the disposal of the excess of unassembled class I subunits by divergence to a non-lysosomal pathway of degradation. A link to the ubiquitin pathway of degradation is suggested by the presence of ubiquitin and ubiquitinylating enzymes in the class I enriched compartments.

We-196

STUDIES ON SOME BOAR SPERM ANTIGENS
BY SPECIFIC MONOCLONAL ANTIBODIES
WITH SPECIAL REFERENCE TO
FERTILIZATION

G. Atanasova, M. Mollova, M. Ivanova
and St. Kyurkchiev

Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Science, Sofia, Bulgaria

To identify antigens involved in gamete recognition sperm monoclonal antibodies have been produced and screened with capacitated boar spermatozoa by ELISA. The immunofluorescent and morphological studies showed that the target epitopes of seven MAbs were plasma-membrane associated antigens, that were exposed during *in vitro* capacitation. Most of capacitated spermatozoa demonstrated granular fluorescence staining along the acrosomal region with these MAbs. The epitope of MAb 3G4 was exposed on the sperm headpiece exhibiting spot-like arrangement. Three MAbs (1F10, 2E2 and 3G4) were found to inhibit boar sperm binding and penetration in homologous *in vitro* fertilization system. These MAbs were selected for further characterization because their cross reactivity with spermatozoa from other species (human, mouse, bull and ram for 1F10, bull and mouse for 2E2 and cat for 3G4) and their biological activity in porcine IVF system.

Data presented provide evidence that MAbs 1F10, 2E2 and 3G4 appear to recognize membrane associated antigens, exposed during capacitation with a role in the fertilization process.

We-197

VARIOUS HISTOTOPOGRAPHY OF SUPPRESSION
AND ACTIVATION OF AMINE-CONTAINING
STRUCTURES OF MESENTERIAL LYMPHATIC
NODES BY CORPUSCULAR AND DISSOLVING
ANTIGENS 7

A. Smorodchenko and V. Sergeeva

Department of Histology, Chuvash State
University, Cheboksary, Russia

The increase as well as the depression of concentration of histamine and serotonin in corresponding regions of mesenterial lymphatic nodes shows that T-dependent antigen suppresses the activity of B-zone follicles. Activating T-dependent paracortical lymphocytic parenchyma. In contrast, B-dependent antigen suppresses T-dependent zone-activating B-dependent central part of the lymphoid follicle.

We-199

POSSIBLE INTERLINK BETWEEN MECHANISMS
OF TISSUE SELF-RENEWAL AND BIORHYTHMS
OF HORMONAL SECRETION AS A CHALLENGE
TO STUDIES IN VITRO ON CELLULAR
INTERACTIONS

V.I.Goudochnikov, M.I.U.M.Rocha

Department of Industrial Pharmacy, Center of
Health Sciences, Federal University of Santa
Maria, Santa Maria, RS, Brazil

Recently we have offered a model considering homeokinetic regulatory systems (F.E.Yates, 1982) in combination with proliferon hypothesis (G.Zajicek, 1977). According to this model, ultradian biorhythms of certain hormones and hormone-like growth factors are intercorrelated in such way as to provide impulsive character of the growth of various tissues. Although in primary monolayer cultures the dispersed cells reassociate to clusters in non-random way, nevertheless well organized system of tissue streaming is unlikely to be restored. Hence, it is necessary to develop special methods of cultivation that permit to evaluate mutually arranged location of cells of different maturity grade and to registrate complex and interconnected processes of cell proliferation, differentiation and death. Besides, the continuous perfusion of cells with culture medium containing oscillatory levels of hormones and growth factors is strictly needed. Some variants of such cell culture systems corresponding to main principles of dynamic histochemistry are presented as general schemes.

We-198

IMMUNOCYTOCHEMICAL CHARACTERIZATION OF
INVARIANT-CHAIN-INDUCED LARGE ENDOSOMAL
COMPARTMENTS

E. Stang and O. Bakke.

Division of Molecular Cell Biology, Dep. of Biology, University of Oslo, Norway.

The major histocompatibility complex class II-associated invariant chain (Ii) is believed to direct newly synthesised class II to endocytic compartments. In transiently transfected cells Ii synthesised at high levels has been shown to induce large vesicular structures which are part of the endocytic pathway. We demonstrate that Ii may induce a similar phenotype in stably transfected human fibroblasts showing that the large vesicles are not lethal. Ultrastructurally, at least three morphologically distinct enlarged compartments could be discerned in the cells. Immunocytochemical labelling indicate that these three compartments may represent early and late endosomes and lysosomes. Internalization of anti Ii antibodies show that Ii may reach the large endosomes via rapid internalisation from the plasma membrane. Internalized protein remained in the enlarged vesicles for 4-6 hours indicating an Ii induced delay in the pathway to lysosomes. Although the large Ii induced vesicles have not yet been seen in professional antigen presenting cells, the Ii induced effects may play a role in regulating the endocytic pathway, creating a special environment for MHC class II to bind antigen.

We-200

PUTATIVE ROLE OF GAP-JUNCTIONAL
COMMUNICATION BETWEEN RAT PITUITARY
CELLS IN THE CONTROL OF PROLACTIN
AND GROWTH HORMONE SECRETION

V.P.Fedotov, V.I.Goudochnikov

Institute of Experimental Endocrinology,
ERC RAMS, Moscow, Russia

Previously, using primary monolayer cultures we have established that octanol - an agent known to block intercellular communication mediated by gap junctions - did not change basal release of immunoreactive prolactin from prepubertal rat pituitary cells but inhibited secretogenic action of dibutyryl-cyclic AMP (DbcAMP). We have demonstrated also that octanol did not influence significantly basal secretion of immunoreactive growth hormone by neonatal rat pituitary cells or secretogenic effect of dibutyryl-cyclic GMP. However, this agent inhibited stimulatory action of DbcAMP and thyroliberin on neonatal somatotrophs. Recently, in the experiments on cultures of anterior pituitary cells from sexually mature rats we have shown that octanol inhibited basal release of electrophoretically determined, ¹⁴C-labelled prolactin, totally blocked secretogenic influence of DbcAMP and phosphodiesterase inhibitor isobutylmethylxanthine, but did not change the stimulatory action of calcium ionophore A23I87 on lactotrophs.

In conclusion, gap-junctional communication may give important contribution to cAMP-dependent mechanisms controlling secretory processes in pituitary cells from rats of different age groups.

**IN SITU ANALYSIS OF MAMMAL SPERM
We-201 CHROMATIN**

C. Pellicciari and M. Biggiogera

Dipartimento di Biologia Animale and Centro di
Studio per l'Istochimica del CNR
Piazza Botta 10, I-27100 Pavia (Italy)

During eutherian mammal spermatogenesis, somatic histones are replaced in the testis by sperm-specific, thiol-rich proteins (the protamines) which allow DNA to be tightly packed into a transiently inactivated dense chromatin. Along the *epididymis* and the *vas deferens*, an additional chromatin stabilization takes place through the formation of disulfide bonds between the thiols of protamine cysteines. The sequential process of chromatin condensation and disulfide cross-linking has generally been thought to give rise to a homogeneously dense complex, whose adaptive role should be to carry the male genome in a protected form up to the penetration into the ooplasm, at fertilization.

In fact, DNA staining and immunocytochemical studies at electron microscopy, as well as quantitative investigations on DNA and protamines in single sperm cells by cytofluorometry and image analysis, provided *in situ* evidence of the presence of local heterogeneities in chromatin stability of mature spermatozoa. In particular, the remnants of protamine free thiols, which were found to persist in spermatozoa from the *epididymis* and the *vas deferens*, could play a key role in the process of chromatin decondensation upon fertilization. The presence of these free thiols would determine a local relative instability of sperm chromatin, which should be also relevant to the experimentally induced nuclear internalization of exogenous DNA into mature spermatozoa.

**We-203 ANALYSIS OF mRNA FOR CLASS I HLA ON
HUMAN GAMETOGENIC CELLS**

D. Fiszer, M. Janitz, K. Michalczak-Janitz, M. Kurpisz. Institute of Human Genetics, Pol. Acad. Sci., Poznań, Poland.

We have studied mRNA expression for Class I on male germ cells by RT-PCR (reverse-transcription polymerase chain reaction), Northern blot analysis and *in situ* hybridization on testicular tissue. mRNA was extracted from sperm or fractionated gametogenic cells (isolated from testis and separated on Percoll gradients) and reversely transcribed. Then cDNA was amplified for specific HLA sequences, i.e. 1151 bp, whole length consensus sequence (HLA, A,B,C) and 418 bp gene fragment coding for alpha 3 domain, transmembrane and cytoplasmic portion of Class I HLA. Sequences of non-classical Class I HLA genes (E, -F, -G) were also studied by RT-PCR. In case of 1151 bp fragment, the obtained results indicate minimal expression of Class I HLA on gametogenic cells. However smaller fragment, common for as well classical and non-classical HLA, was found in quite abundant amounts on gametogenic cell fractions. Further RT-PCR analysis seems to indicate transcripts for non-classical gene sequences but not HLA, -G. Northern hybridization with 669 bp cDNA fragment (spanning for alpha 3 domain, transmembrane, cytoplasmic and 3' untranslated region) resulted in low intensity signal from gametogenic cell fractions and confirmed our findings obtained from PCR for classical Class I HLA sequences. *In situ* hybridization 350 bp cDNA fragment revealed mRNA product in interstitial fragment of testicular section (macrophages, fibroblasts, Leydig cells) and in peribasal compartment (early stages of spermatogenesis) but not on sperm. The minimal expression of classical HLA antigens may create a neutral cover for male reproductive system thereby to prevent immunological response during germ cell differentiation.

**CYTOMETRIC DETECTION AND SORTING
OF UNFIXED APOPTOTIC THYMOCYTES
We-202**

C. Pellicciari^a, A.A. Manfredi^b, M.G. Bottone^a

V. Schaeck^a, S. Barni^a.

^aDip. Biologia Animale and CNR Ctr. di Studio
Istochimica, Piazza Botta 10, I-27100 Pavia; ^bIstituto
Scientifico S. Raffaele, Milano (Italy)

The apoptotic process is marked by cytological peculiarities, such as cytoplasm and organelle condensation, discrete chromatin packaging and successive karyorrhexis, leading to apoptotic bodies formation; furthermore, chromatin DNA is cleaved by endogenous nucleases into oligonucleosome fragments. In the attempt to estimate the frequency of apoptotic cells, either morphology or flow cytometry are generally used, the presence of a sub-G₁ peak in DNA histograms (which relates to DNA loss and/or lower stainability) being the most widely accepted cytometric feature. Relatively lower attention has been given to the morphology-related changes of light scattering values: in fact, apoptotic cells are smaller, denser and exhibit even extensive surface and nuclear blebbing, thus having lower forward scatter (FSC) and higher side scatter (SSC) values, than normal cells. We present a simple and rapid flow cytometric technique for identifying and sorting unfixed apoptotic thymocytes, which combines the assessment of their intrinsic differences in light-scattering with the more traditional detection of the sub-G₁ peak. In dual-parameter scattergrams of FSC vs SSC, apoptotic thymocytes can be identified and quantified, without the need of previous fixation procedures. In addition, apoptotic and non-apoptotic cells can be retrieved by sorting, to be submitted to further biochemical, morphological or immunological analyses. Dual parameter evaluation of DNA content vs scatter values made it also possible to detect apoptotic thymocytes in cell cycle phases other than G₁, following etoposide treatment.

CANCELLED

We-205 CONTROL OF THE N-LINKED GLYCOPROTEIN TRAFFIC AT THE EXIT OF THE ENDOPLASMIC RETICULUM IN INTESTINAL EPITHELIAL CELLS

Ogier-Denis E.¹, Houri J.J.¹, De Stefanis D.², Bauvy C.¹, Baccino F.M.², Isidoro C.² & Codogno P.¹
¹ INSERM U410 Paris France. ² University of Torino, Italy.

We have shown previously that the processing of asparagine-linked oligosaccharides is dependent on the state of enterocytic differentiation of HT-29 cells (a human colon adenocarcinoma cell line). The pivotal observation was an impairment of the trimming of high mannose glycans in undifferentiated HT-29 cells (a human colon adenocarcinoma cell line) (J.Biol.Chem. 263, 6031, 1988; J.Biol.Chem. 265, 5366, 1990). This dramatic decrease in N-glycan processing is dependent on the bypass of the Golgi apparatus by newly synthesized glycoproteins and their subsequent deposit in an acidic degradative compartment. This abnormal glycoprotein traffic was not observed in differentiated HT-29 cells (J.Biol.Chem. 266, 20849, 1991). More recently, we have gained further insights into the lysosomal nature of the degradative compartment and the autophagic-lysosomal pathway that leads to the missorting of glycoproteins in undifferentiated HT-29 cells (Eur.J.Biochem. 205, 1169, 1992; Biochem.Biophys.Res.Commun. 197, 805, 1993). In the present study, we show that: 1-the autophagic sequestration of endoplasmic reticulum markers is concomitant to that of high mannose substituted glycoproteins. 2-the fate of high mannose glycoproteins at the exit of the endoplasmic reticulum in undifferentiated cells (movement to the Golgi apparatus vs autophagic sequestration) is under the control of a heterotrimeric G protein G_i.

We-207 SCHWANN CELLS AND REGENERATION OF PERIPHERAL NERVES
 P.A.R. Ekström and P. Remgard
 Dept. Animal Physiology, Univ. Lund,
 Sweden

A peripheral nerve injury elicits a series of drastic changes in both myelinating and non-myelinating Schwann cells. From being quiescent servers of various types of neurons, the injured Schwann cells rapidly revert to a highly active and proliferating state to provide the best possible conditions for a functional regrowth of the severed nerve cells. This change is often accompanied with a re-expression of proteins related to undifferentiated Schwann cells but also new gene products can be detected. While these and other Schwann cell proteins interact with the growing neurons from a surface-bound position, it can be assumed that there is also a release of important proteins into the extracellular space.

Using cultured sciatic nerves from the adult frog and mouse, a variety of methods showed that the Schwann cells were rapidly and locally activated by a crush-injury. In addition the Schwann cells were found to release several proteins into the culture medium, where they in turn could be further studied and characterized. In the frog nerve a released 70 kDa protein was found to possess an intrinsic serine/threonine protein kinase activity, with no apparent sensitivity to calcium or cyclic nucleotides. Its release was coincident with the onset of Schwann cell proliferation and could play a part in the regeneration process.

We-206 CYTOTOXICITY OF LIPID HYDROPEROXIDES
 Kunio Yagi
 Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505-01, Japan

It is generally accepted that lipid peroxidation is initiated by hydrogen abstraction from polyunsaturated fatty acids contained in lipids and that the lipid radical thus formed is changed to a stable primary product, lipid hydroperoxide, via the lipid peroxy radical. *In vivo* peroxidation also follows this process. When lipid peroxidation occurs in cells, lipid hydroperoxides can be accumulated to some extent in the cells because of their relative stability compared with radical species, and then leak from the cells. Extracellular lipid hydroperoxides in turn attack intact cells, as evidenced by our experimental results that linoleic acid hydroperoxide intravenously injected into a rabbit injured the endothelial cells of the thoracic aorta and that the hydroperoxide added to cultured arterial endothelial cells provoked morphological changes in the cells, inhibition of cell respiration, and leakage of enzymes from the cells. Thus, lipid hydroperoxides are regarded as a transmitter of lipid peroxide-mediated cell injury. It was noticed, however, that the cytotoxic effect of hydroperoxide can be prevented by the presence of suitable antioxidants, indicating that the toxicity is due to radical species. Relating to this problem, our recent experiment demonstrating the generation of hydroxyl radicals from linoleic acid hydroperoxide in the presence of ferrous iron should be emphasized.

Hydroxyl radical generation was also found with oxidatively modified low density lipoprotein in the presence of ferrous iron, and the ferrous iron could be replaced by the ferric iron-epinephrine complex. This result would explain at least in part the mechanism of catecholamine-induced atherosclerosis and even stress-induced atherogenesis.

We-208 REGULATION OF BIOSYNTHESIS OF MAMMALIAN MITOCHONDRIAL ATP-SYNTASE (ATPase)
 J. Houšek^a, U. Andersson^b, P. Tvrdfk^{a,b}, J. Nedergaard^b and B. Cannon^b

^aInstitute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic; ^bThe Wenner-Gren Institute, Stockholm University, S-106 91, Stockholm, Sweden.

Biosynthesis of mammalian mitochondrial ATPase depends on both the nuclear and mitochondrial genes. All subunits of the F₁-catalytic part (α , β , γ , δ , ϵ , inhibitor protein IF1) and six subunits of the H⁺-translocating membrane-spanning Fo-part (b, c, d, e, F6, OSCP) are of nuclear origin, two other Fo subunits, a and A6L are mitochondrially encoded. The amount of ATPase largely varies in different tissues and species, and changes during ontogeny or in response to adaptation. Using the model of selective reduction of ATPase content in thermogenically active brown adipose tissue a good correlation was found between decreased synthesis of ATPase and the mRNA levels of c subunit of Fo. In contrast, the other nucleus-encoded F₁ subunits (α , β , γ) and Fo subunits (b,d), as well as mitochondrially-encoded subunits exhibited 20-100-fold relative overexpression. Likewise, only the changes in subunit c transcripts correlated with the ontogenetic changes in the content of ATPase and with species-specific differences.

The results indicate that transcriptional control of two *c*-Fo genes (coding for the same protein with different import presequences) and respective synthesis and availability of the extremely hydrophobic, DCCD-reactive subunit c is crucial for regulation of enzyme biosynthesis. This is in accordance with well established absolute requirement of subunit c for assembly of the Fo part of the ATPase complex.

We-209 CONTROL OF α (1-2) FUCOSYLTRANSFERASE IN THE MOUSE UTERINE LUMINAL EPITHELIUM BY OVARIAN STEROIDS

S. Kimber, S. White and A. Cook

School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK

We previously reported that the H-type-1 carbohydrate antigen, expressed on the luminal epithelium of the murine uterus, is involved in attachment of the embryo at implantation. The major evidence for this is the inhibition of embryo-attachment to uterine luminal epithelial monolayers by H-type-1 pentasaccharide or an antibody to this sugar epitope. We have now partially characterized the major component carrying this antigen in the uterus as a 120-130 kD trypsin-insensitive glycoprotein bearing α -linked carbohydrate chains. This is in contrast to related fucosylated (Le) antigens which are present on multiple epithelial glycoproteins. Expression of the H-type-1 epitope on the luminal epithelium is under the control of ovarian steroids. Using ovariectomized mice given steroid supplements we have now demonstrated that regulation of the appearance of the epitope occurs by estrogenic stimulation of α (1-2)fucosyltransferase activity in the luminal epithelium and its inhibition by progesterone. Changes in the activity of α (1-2)fucosyltransferase precede the appearance and disappearance of the H-type-1 epitope during pregnancy and the estrus cycle. We are now investigating control of the murine α (1-2)fucosyltransferase gene in the uterus using northern blotting and *in situ* hybridization.

We-211 ANALYSIS OF RETROVIRAL POLYPROTEIN TARGETING AND VIRION ASSEMBLY
H.-G. Kräusslich¹, R. Welker¹, M. Fäcke¹, A.-M. Heuser¹, and A. Janetzko²

(1) Deutsches Krebsforschungszentrum, D-69009 Heidelberg;
 (2) Max-Planck-Institut für Zellbiologie, D-6802 Ladenburg. GERMANY

Retroviral morphogenesis involves assembly of the structural Gag- and Gag-Pol polyproteins within the infected cell with subsequent budding of the virion from the plasma membrane and proteolytic cleavage by the viral proteinase. The glycoproteins of the viral envelope (Env) are translated on membrane-bound ribosomes and processed and transported via the secretory pathway to the plasma membrane. Synthesis of the internal structural proteins (Gag- and Gag-Pol), on the other hand, occurs on cytosolic polyosomes.

Previously, we had shown that an internal deletion of 84 amino acids in the N-terminal matrix (MA) domain of the HIV-1 Gag polyprotein all but abolished release of extracellular virions and led to assembly and budding of viral particles into the cisternae of the endoplasmic reticulum (ER). This phenotype closely resembled the assembly pathway of murine intracisternal A-type particles (IAP), a class of defective endogenous mouse retroviruses that assemble and bud into ER cisternae and remain there as immature virus particles. Sequence analysis of several IAP genomes indicated sequence homologies with other retroviruses in most viral genes with a conspicuous absence of similarity in the N-terminal region of gag, corresponding to the MA domain.

To analyze the role of specific viral protein domains in protein targeting and viral assembly we constructed mammalian expression vectors containing the gag gene of the murine IAP element MIA14 or chimeric genes derived from its sequence. Expression of the MIA14 Gag protein in COS cells yielded abundant IAPs within the ER cisternae indicating that this phenotype is not dependent on expression in murine cells. Deletion of an N-terminal hydrophobic sequence that had been postulated to serve as an ER targeting sequence abolished IAP formation and yielded mainly cytosolic protein. Replacing the N-terminal sequence of the MIA polyprotein by the N-terminus of the Src protein, which had been shown to function as a dominant membrane targeting signal in a heterologous context, redirected polyprotein targeting in transfected cells and yielded assembly and release of chimeric virus particles from the plasma membrane of these cells.

We-210 INTERCELLULAR SIGNALS CONTROL THE ANTHOCYANIN PATTERN IN MUSTARD COTYLEDONS

P. Nick^a, B. Ehmann^a, M. Furuya^b, E. Schafer^b

^aInstitut für Biologie II, 79104 Freiburg/Br., Germany, ^bHitachi Advanced Research Laboratory, Hatoyama, Saitama 350-03, Japan

Since higher plant cells have a fixed location, pattern formation must be based upon differentiation of individual cells, guided by intercellular signals. This was investigated using anthocyanin pattern in cotyledons of white mustard (*Sinapis alba L.*) as a model system. The expression of this pattern is triggered by the plant photoreceptor phytochrome. Microbeam irradiation of different leaf domains in combination with *in situ* hybridization of chalcone synthase mRNA (an early molecular marker for anthocyanin synthesis) were used as approaches. During early stages of pattern formation individual cells exhibited all-or-none responses with a resultant stochastic, patchy pattern. These responses of individual cells are subsequently integrated by long range inhibitory signals. The resulting final pattern develops in an ordered and gradual fashion, if analyzed on the whole-organ level. A similar mechanism has been found for microtubule reorientation in grass coleoptiles (a response controlled by a blue-light receptor). Stochastic all-or-none responses of individual cells and inhibitory intercellular signals are discussed with respect to a general understanding of plant morphogenesis.

We-212 CHICKEN MAR BINDING PROTEIN p120 IS IDENTICAL TO HUMAN HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN (hnRNP) U

J.P. von Kries^a, F. Buck^b and W.H. Sträling^a

^aInstitut für Physiologische Chemie; ^bInstitut für Zellbiochemie und Klinische Neurobiologie; Universitäts-Krankenhaus Eppendorf, Martinistr. 52, D-20246 Hamburg

We have previously identified two proteins from chicken oviduct nuclei that specifically bind to matrix/scaffold attachment regions (MARs/SARs). Here one of these proteins, named p120 due to its apparent molecular weight, is purified to near homogeneity and shown to be identical to a previously described component of heterogenous nuclear ribonucleoprotein particles, hnRNP U, on the basis of amino acid sequence analysis of tryptic peptides. p120 binds to multiple MAR fragments provided they have a minimal length of approximately 700 bp. Binding of MAR fragments is specifically competed by homoribopolymers poly(G) and poly(I), which form four-stranded structures. Our results suggest that p120/hnRNP U may serve a dual function, first as a component of hnRNP particles, and second as an element in the higher-order organization of chromatin.

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HORMONES AND
NEUROTRANSMITTERS RELEASE:
FOUR MECHANISMS OF SECRETION

Yves DUNANT

*Département de pharmacologie, CMU,
CH-1211-Genève-4, Switzerland*

Distinct mechanisms operate in different secreting systems, they are 1) free diffusion through the plasma membrane; 2) exocytosis resulting from fusion of a secretory granule with the plasma membrane; 3) fleeting release from a granule through a transient pore without full fusion; 4) release through a specialised plasmalemmal molecule such as the mediapophore. The latter mechanism is proposed to operate in rapid synapses in which the neurotransmitter is emitted as an abrupt chemical impulse of quantal composition. There, release is momentarily signalled in the plasma membrane by large intramembrane particles. Synaptic vesicles are also essential for regulation of this type of release. They fuse with the plasma membrane only late after activity and seem to be involved in calcium sequestration and extrusion.

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DEVELOPMENT OF HIV-1 PROTEINASE
INHIBITORS AS ANTIVIRAL DRUGS

J. Mous

Pharmaceutical Research, F.Hoffmann-La Roche AG, Basel, Switzerland.

The rational development of antiviral chemotherapeutic compounds is based on the development of agents that are directed at specific steps of the viral replicative cycle that are not shared by the host.

Recently, a new class of antiretroviral compounds entered clinical trials, inhibitors of HIV-1 proteinase. These inhibitors block late steps in the replication cycle, viral polyprotein processing and maturation. Chronically infected cells when treated with this class of inhibitors start to produce immature, non infectious virus progeny. Hence, treatment leads to a blockade of virus spreading. Saquinavir (Ro 31-8959), an optimised representative of this inhibitor type is currently in Phase II/III trials. First results confirm its antiviral activity also in patients. Virus with reduced sensitivity to Saquinavir could be obtained in cell culture after exposure to increasing amounts of the drug. The importance of this finding in the light of the ongoing clinical trials will be discussed.

Th-1 DETERMINED PROGRAMMED DESTRUCTION OF GAMETOPHYTE CELLS DURING FORMATION OF SPOROPHYTE OF A LEAFY MOSS

V.I. Matasov

Department of Plant Ecomorphogenesis, Institute of Ecology of the Carpathians of the Academy of Sciences of Ukraine, 290000 Lviv, Stepanika Street, II, Ukraine

Disruption of embryotheca is peculiar characteristic in leafy mosses. It performs while differentiated growth is accompanied by destruction of cells as a result of sporophyte development. Growth of sporogon of *Physcomitrella patens* (Hedw.) B.S.G. and *Pottia davalliana* (Sm.) C. Jens. is concomitant with its penetration by means of lysis of surrounding cells at first in base of archegonium then in tissue of a top of gametophyte. Widening of middle part of archegonium under influence of developing sporophyte and its general growth by means of cell divisions and cell increase form embryotheca. Further growth of sporophyte results in disruption of upper part of embryotheca - calyptra from lower one - vaginula. Process of disruption of embryotheca of *Pottia davalliana* is followed by formation on its narrowing part a girdle zone which consists of 4 rows of small cells with thin, frail walls. The zone of disruption is limited below and above by large, unable to disrupt cells of vaginula and calyptra correspondingly. Existence of anomaly of rupture of embryotheca in mutants that takes place when sporophyte breaks through the top of embryotheca on the outside, suggests genetic determination of destruction and death of cell of gametophyte.

Th-3 ULTRASTRUCTURE OF LEAF MESOPHYLL IN *Pseudotsuga menziesii* VAR. *menziesii*

G. Corneanu^a, C. Crăciun^b, V. Crăciun^b

^aGenetics Sect., Craiova University, 1100 - Craiova; ^bExperimental Biology Dept., Babes - Bolyai University, 3400 - Cluj, Romania.

Mature leaf has a bifacial, dorsiventral structure. Unistratified epidermis, present cells with the walls strong sclerification, with a thick cuticle. The stomata, rarely, are disposed gentle on the lower epidermis. Hypodermis, in fragments, is formed by a single cells layer, with the sclerified walls, disposed at the middle of the leaf (8 - 10 cells under upper epidermis, 2 - 4 cells under lower epidermis), under resin - ducts (2 - 6 cells) and, dependent on genotypes, at the leaf ends.

The parenchyma cells present the walls straight or slightly undulated, without folding. Palisadic parenchyma (2 cells layers) is formed by elongated cells with small spaces between them, rich in cytoplasm, with numerous cellular organelles. Big nucleus, disposed in the middle of the cell, present, a fine blocks of heterochromatin. The chloroplasts present the rows of 2 - 5 long thylakoids, unorganized in grana. Between them exist numerous plastoglobuls, and rarely starch grains. Around the chloroplasts there are clusters of mitochondria and dictiosomes.

The lacunar parenchyma cells from the first layer under lower epidermis present a similar structure. The cells from other three layers present numerous synthesis granules thinly dispersed in them, accumulated then in big granules and in vacuoles. The nervure is surrounded by an evident endodermis, and present vessels, tubes, cambium and parenchyma. In these cells there are numerous cellular organelles. The proplastids have spherical form, with 1 - 2 thylakoids disposed peripheral and plastoglobuls. The resin - ducts present the cells with thicker walls, lined inside by secretory cells disposed on one layer.

Th-2 CYTOSKELETON STRUCTURES CAN ENCODE POSITIONAL INFORMATION

B. V. Sorochinsky

Department of Biophysics and Radiobiology,

Institute of Cell Biology and Genetic Engineering, Ukrainian Academy of Sciences, 148 Zabolotnogo st., Kiev, 252143, Ukraine.

Patterning in plants is encoded in term of positional information but nothing is known about the physical nature of this information. I hypothesised that cytoskeleton structures could encode the positional information. The idea was confirmed on the investigation of the duckweed plant's (*Spirodela polyrrhiza*) development. There is such important morphological feature for this plant as temporal appearance of left (-L) or right (-R) daughter forms. The subpopulation of L-forms always generate left forms first as well as subpopulation of right forms always generate R-forms first. This feature was split in first generation after repeated treatment with high concentrations of colchicine (0.01M-0.001M). This results demonstrate the cytoskeleton participation in the encoding of positional information.

Th-4 STRUCTURAL CHANGES IN ENHANCED AXILLARY BUD INITIATION DURING TRANSITION TO INFLORESCENCE FORMATION

J.T.P. Albrechtová^a, F. Seidlová^b

^aInstitute of Experimental Botany, Acad.Sci.Czech Rep., Ke dvoru 15, Praha 6, Czech Republic, ^b Biological Faculty, South Bohemian University, Branišovská 31, České Budějovice, Czech Republic

In vegetative shoot apices buds are formed in the axils of leaf primordia with a delay of several plastochrons. The inner tunica and the outer corpus participate in bud initiation. The newly formed bud primordium consists of compartments with different cell division rate, orientation of cellular growth and cytology. The outer tunica cells divide anticlinally at a high rate and show the most meristematic features. The bud primordium is separated from the parenchyma cells of the internode by thick-walled, mostly flattened cells in the direction towards future possible branch outgrowth. Between those two compartments another group of cells with alternating orientation and slightly advanced differentiation are formed.

At transition to flowering bud initiation starts soon after formation of leaf primordium. Cell division rate becomes higher in all compartments. The boundary between the growing bud and the internode becomes less distinctive. These changes indicate a shift in cell-to-cell communication inside the bud and between the bud and the rest of the plant body. Distribution of primary and secondary plasmodesmata and cell differentiation in terms of the vacuolar system formation is compared in different compartments of the buds in the shoot apices of *Chenopodium rubrum* induced to flowering by short-day photoperiods and in non-induced vegetative apices.

Th-5 SUBCELLULAR ROOT MERISTEM MORPHOLOGY IN
MAGNETIC FLUIDS

Gheorghe Butnaru^a, V. Bujoreanu^b, D. Terteac^b

^a University of Agricultural Sciences of Banat County,
Timișoara, Romania.

^b Institute of Genetics Chisinau, Rep. Moldova

The ultrastructure cell morphology of root tips grown for 12h in Magnetic Fluids mixtures (MFs; Fe_2O_3 and Fe_2O_4 g/cm³; $E1=0.35 \times 10^{-4}$; $E2=0.70 \times 10^{-4}$; $E3=1.05 \times 10^{-4}$) revealed specifically subcellular modifications in comparison to control (H_2O -distilled). The main exception in E1 consists of a high physiological activity due to a huge endoplasmic reticulum (ER) associated with ribosomes (Rs). In the cytoplasm there are many cis, medial and trans vesicles. The mitochondrion consists of small electron-dense mitochondria, with strongly formed cristae. The cell ultrastructure was completely changed when the roots' growth took place in E3. Two types of cells were observed: the "normals" were rather unfrequent (0.2-3%) and "ghostly" strongly vesiculated, as empty cells without caryolymph, ER, Rs and organelles. The cells with a strongly modified structure resemble the senescents or those affected by pathogen agents.

Th-7 ACETYLCHOLINESTERASE ACTIVITY IN STOMATA
OF MARCHANTIA POLYMORPHA L

D.J. Smoliński and A. Górska-Brylass

Institute of Biology, Copernicus University, Toruń, Poland

The acetylcholinesterase activity has been found in stomata of *Marchantia polymorpha* L. The cytochemical methods used after Karnowsky-Rools and Koelle (modified by Tsuji) showed that the product of enzymatic reaction of AChE (electron dense precipitate of copper ferrocyanide) accumulates in the stomata mainly on the surface of guard cells walls which surround the pore.

The inhibitors of animal cholinesterases eserine and neostigmine inhibited AChE activity in stomata. High concentration of substrate (acetylthiocholine iodide) inhibited this reactions.

The specificity of the cytochemical methods has been verified by X-ray microanalysis EDS of elements forming the product of enzymatic reaction. In both cases, it was shown that the product contained cooper and iron i.e. elements forming exact product of enzymatic reactions indicating AChE activity.

Th-6 MOLECULAR ANALYSIS OF CYTOKININ ACTION IN
PHYSCOMITRELLA PATENS

R. Reski, S. Kruse, B. Kasten, K. Reuter, M. Wehe, M. Faust, G. Gorr, W. O. Abel
Institut f. Allgemeine Botanik, D-22609 Hamburg
Germany

Within hours cytokinins induce cellular differentiation in the moss *Physcomitrella patens* as well as division of macrochloroplasts in a particular *Physcomitrella*-mutant. Concomitantly, cytokinins promote maturation of complex plastid transcripts and a transient occurrence of plastid polypeptides. These cytokinin-induced molecular changes interfere with other internal and external stimuli, e.g. blue light and endogenous oscillators. Detailed analyses utilizing two-dimensional electrophoresis (IEF/SDS-PAGE) reveal a cascade of plastid proteins underlying chloroplast division in this particular mutant. Identification of these proteins by Western-analyses and microsequencing will be reported. Restriction enzyme- and Southern-analyses gave no hints for mutations in the plastid DNA of this chloroplast mutant but revealed its methylation around an open reading frame (ORF), possibly encoding a zinc-finger protein. Expression of the protein and analysis of its function will be reported. Four cDNAs representing novel genes have been isolated by molecular subtraction. Their expression is developmentally regulated and respond to cytokinin within one hour. Expression of these genes and behaviour of the mutant macrochloroplast was analysed in somatic hybrids and in transgenic plants overproducing cytokinin.
Acknowledgements: This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Commission of the European Union.

Th-8 ELECTRIC CABLE SPACE CONSTANT DETERMINES
THE FUNCTIONAL MODULE LENGTH OF FUNGAL HYPHAE

T.V. Potapova*, L.Ju. Boitzova*, K.B. Aslanidi~
"The A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University,
119899 Moscow; ~Laboratory for Cellular Biophysics, Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, 142292 Moscow Region, Russia.

The association of the idea of intercellular electric communication via cell-to-cell permeable junctions (PJ) with P.Mitchell chemiosmotic concept for plasma membrane allows to predict the *N.crassa* growing hyphae self-organization.

We discovered cable construction of the hyphae and demonstrated the energy transferred via ionic fluxes from the proximal compartments to the apical ones. Energetic and metabolic communications should be effective at the dimensions equal (or less) to the electric cable space constant.

The growing hyphal tip of 0.6-0.8 mm is a subsystem of the specialized cells (functional module) possessing of all components needed for the normal growth.

The electrical cable space parameters have really determined the dimensions of the growth unit in *N.crassa*. The ratio of the full length of hyphae (including all branches) to the electric cable space constant is always less or equal than the number of tips. The distance between branches is always no more than electric cable space constant.

We are sure that the all tissues have a modular construction. We suppose that cell specialization takes large part in the functional activity of all multicellular systems and biochemical specialization is realized not only for membrane processes.

Th-9 LATERAL ROOT INITIATION BY MEANS OF ASYMMETRICAL TRANSVERSE DIVISIONS OF THE PERICYCLE CELLS.

P.J. Casero, I. Casimiro and PG. Lloret

Departamento de Ciencias Morfológicas y Biología Celular y Animal,
Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz,
Spain.

Asymmetrical transverse divisions have been analyzed in the pericycle cells of roots of *Allium cepa*, *Raphanus sativus*, *Helianthus annuus*, *Zea mays* and *Daucus carota*, between the proximal limit of the apical meristem and the transverse level where the pericycle cells undergo pericinal divisions. Asymmetrical transverse divisions always occur nearly simultaneously in two neighbouring large and highly vacuolated pericycle cells in the same column. Then, two very short pericycle cells are produced, flanked by two longer ones. In onion, radish and sunflower, these asymmetrical transverse divisions occur in front of the xylem, while in corn and carrot, they occur close to the phloem. These results are very noticeable because in the three former species, lateral roots appear in front of the xylem while in the two latter ones, they appear close to the phloem. Later, in most proximal sections, new very short cells are added to the first pair by asymmetrical transverse division of the longer pericycle cells located above and below, respectively. Further from the apex, one of these short cells divide periclinally. All these results, strongly suggest that lateral root initiation occurs earlier and nearer the apex than have been previously reported, when two neighbouring large and highly vacuolated pericycle cells undergo asymmetrical transversal divisions giving rise to two adjacent very short pericycle cells.

Th-10 THE SUBSTRUCTURE OF MERISTEMATIC CELLS FROM THE TERMINAL BUDS OF PLANTS

N. Sytnyanskaya

M.M. Grishko Central Botanical Garden Ukr. Acad. Sci., Timiryaziv-ska str., 1, 252014, Kiev-14, Ukraine

The structural and functional organization of meristematic cells of terminal buds in woody plants (*Juglans regia* L. and *Acer platanoides* L.) has been studied from dormancy breaking to the evident growth of juvenile sprout. A forced dormancy is an increased synthesis of lipid material that is expressed in the accumulation of lipid bodies (LB) in the cytoplasm. Meristematic buds are almost completely filled with LB being structurally similar to a storage tissue, that appears to be of importance in the inhibition of a premature proliferation of meristem cells.

Intensive mobilization of reserve lipids material concentration in lipid bodies characterizes period of spring growth. LB degradation occurs due to derotation of dictiosomes of Golgi apparatus capable of liplytic enzymes. Lipid material hydrolysis is completed by LB transformation into typical plant vacuole. In some cases numerous and variable in form and size membrane formations are observed in the lysis nidus of LB matrix. The data allow to suppose that LB hydrolysis products are utilized by cell for membrane structures formation of cytoplasm in the period of spring growth.

Th-11 DIFFERENTIAL EXPRESSION OF HOMOLOGOUS mRNA TO UBIQUITIN-ACTIVATING ENZYME DURING POLLEN DEVELOPMENT OF *BRASSICA NAPUS* L. AND *ARABIDOPSIS THALIANA* L.

T. Havlický^{1,2}, Hause¹, K. Angelis², H. Körber², B. Hause¹, P. Pechan²,
A.A.M. Van Lammeren¹

¹Dept. Plant Cytol. & Morphol, Wageningen Agricult. Univ.
Arboretumlaan 4, NL-6703BD Wageningen, The Netherlands;

²Max-Planck-Instit. Biochem., Am Klopferspitz 18a, D-82152
Martinsried, Germany; ¹permanent address: Institute of Experimental
Botany, Sokolovská 6, CZ-772 00 Olomouc, Czech Republic

The expression of mRNA homologous to yeast UBA1 gene for ubiquitin-activating enzyme (E1) was investigated during pollen development of *Brassica napus* L. and *Arabidopsis thaliana* L. using non-reactive *in situ* hybridization techniques. E1 is an enzyme important in the ubiquitin-dependent cellular processes (e.g. proteolysis).

In both species the same specific pattern of gene expression was detected. There was no expression during the microspore stage and in the vegetative cell of the pollen whereas the generative cytoplasm of young sperm cells exhibited a strong hybridization signal of the probe. Possibly the differential expression of E1 mRNA leads to the degradation of various proteins in the generative and sperm cells and is involved in the typical differentiation of those cell.

CANCELLED

Th-13

PROTOPLAST CULTURE FROM SECONDARY
EMBRYOGENIC CALLUS OF WINTER WHEAT
(TRITICUM AESTIVUM L.)

Maystrov P.D., Kuchuk N.V., Bannikova V.P.,
Gleba Yu.Yu.

Institute of Cell Biology & Genetic Engineering, Zabolotnogo str.,
148. Kiev 252022, Ukraine

Four cultivars of winter wheat - Mironovskaja 808, Kashutka, Kievskaja semidwarf and Lutescens 7 were tested in this experiment. Immature seeds of field-growth plant were collected on 12-14th day after pollination and used for the establishment of callus culture. The immature embryos were dissected from seeds and placed onto MS nutritional medium with 2 mg/l 2,4-D at 27°C in the dark. After 40 days in culture the embryogenic looking calli were formed and then transferred onto modified MS medium with 2 mg/l 2,4-D and 0,1 mg/l kinetin. After 10 months in culture, cell callus line MP1 (cv. Kashutka) with good embryogenic potential were established. Calli of this line MP1 were used for protoplast isolation. They were preincubated in W5 medium for 2h and then incubated with digestion solution for 14-16 hours. Protoplasts isolated from cell the line MP1 could divide and form microcolonies on liquid MS medium with 1 mg/l 2,4-D, 0.2 mg/l kinetin and 0.2 mg/l NAA. First division of protoplasts occurred within 10-14 days after the beginning of culture. These protoplasts were able to divide with plating frequency scored as 35-55%. Protoplast-derived microcolonies (up to 6% of the divided protoplasts) were formed. A white, compact and nodule-like calli were obtained from the microcolonies after two months in culture. Embryo-like structures have been observed on the surface of this calli during next month of culture.

Th-15

HISTOLOGICAL AND ELECTRON-MICROSCOPIC
ASPECTS OF TUMOR TISSUE CELLS INDUCED
BY WILD AND MODIFIED STRAINS OF *Agro-*
bacterium tumefaciens

A. Brezeanu, Alex. Calin, P. Cornea, D. Cărciumarescu, M. Costache

Laboratory of Genetic Engineering and Morphogenesis,
Institute of Biology, Bucharest, Romania

Tumoral tissue cells induced by infection of *Solanum tuberosum*, *Populus alba*, *Vitis vinifera*, and *Nicotiana tabacum* plants with wild and kanamycin resistant strains of *Agrobacterium tumefaciens* were analyzed by histological and electronmicroscopical techniques in order to characterize the wound response as well as the possible differences of the explants cells reaction after infection with *Agrobacterium*. These studies could also indicate the nature or the possible changes at the cellular level in the transgenic plants. The infection with *Agrobacterium* strains induced pronounced histological modifications within few days, including extensive cell proliferations, "meristemoids" differentiation as well as numerous nodules with core of tracheids surrounded by cambial like layers. At the marginal area caulinary buds appeared frequently. The *C₅₁* strain was able to induce rooty tumours at *V. vinifera*, a *tum* mutation which affects the gene for the biosynthesis of the cytochalinin precursor. At the cellular level nucleus with extremely lobated aspect is common. These is followed by nuclear fragmentation which finally could determine the epigenetic reversible modifications. In the same time, in tumour cells of *S. tuberosum* induced by kanamycin resistant strains numerous membranal formations differentiated from external membrane of nuclear envelope which we explain as a stress effect of the infection.

Th-14

OBTAINING HERBICIDE-RESISTANT PLANTS

B. Levenko, I. Stekhin, V. Mar'yushkin, M. Rubtsova,
A. Melnik, A. Sayaz, G. Bileka, N. Bogomolova

Institute of Plant Physiology & Genetics, Ukrainian Acad. Sci., Kiev,
Ukraine.

Methods of genetic transformation of sugarbeet, soybean, potato, buckwheat were worked out. For obtention of herbicide resistant transgenic plants different types of explants were inoculated by *A. tumefaciens* strains, carrying plasmids with NPTII gene, mutant aro A gene, encoding glyphosate resistant EPSP-synthase, and bar gene, encoding phosphinothricine acetyl transferase.

Molecular analysis of green plants after canamycin selection was done by dothybridization using as a probe DNA fragments carrying mutant aro A and bar genes. DNA of the plants that gave positive autoradiographic response was further blot-hybridized with the same [532 OP]-probe.

Transgenic soybean, potato, sugarbeet plants, carrying mutant aro A gene and buckwheat plants carrying bar gene both under 35S promoter in pGV-941 vector were obtained.

More than one thousand regenerants of soybean were tested in soil for herbicide resistance. 33 plants survived spraying by glyphosate concentration that totally inhibited the growth of control plants. Tolerance to this glyphosate concentration was confirmed in second and third generations of transgenic soybean plants.

Th-16

Agrobacterium tumefaciens MEDIATED
TRANSFORMATIONS IN *Brassica napus*
HYPOCOTYL SEGMENTS: OPTIMIZATION OF THE
CONDITIONS AFFECTING TRANSFORMATION
EFFICIENCY

Š. B. Šebestianová

Dept. Genet. Microbiol., Charles Univ., Prague, Czech Republic

In transformation experiments there is usually very sad difference between the small number of successfully transformed plants and the big number of transformed calli which are not able to produce any shoots. Also nonrooting transformed shoots can occur quite often. That is why the determination of the optimal conditions to regenerate from the explants into the mature plants is important. For some plant species are these conditions well established but for others, even using of different varieties, can require at least different hormone combinations to induce the regeneration.

Agrobacterium tumefaciens SLJ 1111 containing NPT II and GUS activity was used for transformation of segments from young hypocotyles in *Brassica napus* cv. Westar.

The dependence of the transformation efficiency on the age of used seedlings, length of the segments, tobacco feeder layer, concentration of the Agrobacterium, duration of the exposure of the growing hormones, silver nitrate effects and various ways of the shoot regeneration (using of the solid or liquid medium or cultivation on the membrane rafts) were compared. Rooting problems of the shoots have been solved too.

Th-17 PROTEIN PATTERNS OF TRANSGENIC SOYBEAN CARRYING DIFFERENT MORPHOREGULATORY GENES FROM T-DNA pTi C58

E. Hlinková

Department of Genetics, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia

Transgenic soybean with incorporated genes from the T-DNA of plasmid pTi C58 and morphoregulatory genes 1,2,5 and 4 from the binary vector plasmids pCB 1349 resp. pCB 1334 showed significant differences on both, molecular and morphological levels. PAGE of native proteins from the leaves showed quantitative and qualitative changes in the protein patterns. Due to the expression of genes from T-DNA plasmid pCB 1349, new proteins were detected. One of them has a slight peroxidase activity. Something like that protein have been found in transgenic soybean carrying gen 4 /transformation with the plasmids pCB 1334/. SDS-PAGE confirmed changes in protein spectrum coded by genes 1,2,4,5. Transgenic plants carrying gene 4 have had a low level of chlorophyll a. Leaves and internodes of transgenic plants were reduced more than 50% to compare with the control plants.

AGROBACTERIUM - MEDIATED TRANSFORMATION OF PEAR CULTIVARS (*PYRUS COMMUNIS* L.).

Th-18 S.M.Merkulov, T.P.Pasternak, A.Golovko, Yu.Yu. Gleba

Institute of Cell Biology and Genetic Engineering, Academy of Sciences of the Ukraine, Kiev, Ukraine.

A system was developed which allows the transfer of foreign genes into pear cultivars (*Pyrus communis* L.). The leaf discs (cv. Veajniza) were inoculated with *A. tumefaciens* strain A 281 carrying a binari vector plasmid pCV 730, which contains a nopaline syntase (NOS) promoter driven neomycin phosphotransferase (NPTII) gene. The inoculated leaf discs, precultured for 5 days on non-selective shoot regeneration medium, formed light-green calli on selection medium, containing 50 µg/ml kanamycin. These calli developed into transformed shoots at a friquency of 45% on a second selection medium, containing 25 µg/ml kanamycin. The selected shoots were multiplied on shoot proliferation medium in the presence of kanamycin. All such shoots were resistant to kanamycin and expressed varying levels of NPTII activity. Leaf discs prepared from transformed plants, when put through the second selection cycle on kanamycin, formed callus and adventitive shoots.

Th-19 APOGAMY IN THE DIPLOPHASE OF THE MOSS *Pottia intermedia* AS EXPERIMENTAL MODEL OF EPIGENETIC INHERITANCE

R. Ripetskyj, N. Kit

Department of Plant Ecomorphogenesis, Institute of Ecology of Carpathians of the Academy of Sciences of Ukraine, 290000 Lviv, Stepanic Street, 11

Mosses are suitable for experimental studing cell inheritance of nongenotypic changes because their gametophyte can regenerate directly from single diversely differentiated cells. The capacity for apogamy of aposporic gametophyte derived from sporophyte of *P.intermedia* has been shown to be stably retained through vegetative generations, state of the capacity being transmitted by individual isolated cells. Apogamy is absent however in colchicine diploids and in somatic hybrids obtained by fusion of protoplasts of haploid protonema cells. Normal sporophyte of *P.intermedia* gives rise to apogamous or nonapogamous clones according to the site of regeneration. Among 45 clones regenerated from cells of outer spore sac walls of normal premeiotic capsules 26 (58%) were nonapogamous and 19 (42%) apogamous. Among 76 clones obtained from sporophyte tissues nonadjacent to the sporogenic tissue only 4 (5%) were nonapogamous. The loss of the capacity for apogamy must have been connected with an influence of premeiotic sporogenic tissue. Approximately equal quantity of different clones may be indicative of the loss of capacity for sporophytic morphogenesis being a gradual process.

Th-20 INTERSPECIFIC SOMATIC CYBRIDS OBTAINED BY PROTOPLAST FUSION BETWEEN *SOLANUM TUBerosum* L. AND γ -IRRADIATED WILD *SOLANUM* SPECIES

D. Yevtushenko, V. Sidorov

Institute of Cell Biology & Genetic Engineering, Zabolotnogo 148, Kiev-GSP-22, 252022, Ukraine.

Cybrid potato plants were produced using modified polyethylene glycol fusion protocol.

Mesophyll protoplasts from the chlorophyll deficient plastome mutant of *Solanum tuberosum* L. cv. 'Zarevo' served as recipients. *S. acaule* Bitt., *S. berthaultii* Hawk., *S. cardiophyllum* Lindl. were used as organelle donors after irradiation of mesophyll protoplasts with the lethal γ -ray dose (1000 Gy).

The cells were immobilized in Ca-alginate and cultivated in liquid medium. This procedure significantly increased cell survival and yield of putative cybrids.

Selection of the cybrids was based on their ability to form green calli on the solid medium with low level of carbon sources. Regeneration of the selected green calli was obtained on the modified Shepard's medium containing 0.1 mg/l IAA, 0,05 mg/l GA₃ and 1 mg/l zeatin. The obtained cybrid plants were morphologically similar to *S. tuberosum* L. The detailed biochemical analysis will be presented elsewhere.

AMITOSIS AND MORPHOGENIC CHANGE IN PLANTIS
CAUSED BY ETHEPHON

Th-21 T. Selga & M. Selga
Institute of Biology, Academy of Sciences
of Latvia, 3 Miera st., LV 2169 Salaspils,
Latvia

Thirty day old cucumber (*Cucumis sativus L.*) plants were sprinkled with a water solution of Ethephon (2-chloroethylphosphonic acid) at a concentration of

4×10^{-3} M. It caused a two stage morphogenic change - from inhibition of growth to promotion of side shoot development, bulging of mesophyll tissues of mature leaves of the main stalk, decrease in volume and increase in number of the palisade parenchima cells, close contacts of DNA containing organelles and signs of movement of genetic material. Mechanisms of division of the nuclei, chloroplasts and mitochondria are similar. In the many palisade parenchima cells the amitotic division of the nuclei proceeds by the following stages: the nucleus stretches, develops a constriction, that divides the nucleus in two unequal parts; it bends in two and divides in the place of constriction. In the same cells, fission by strangulation of chloroplasts and mitochondria can be observed. Activated budding of the all three DNA containing organelles as modification of amitosis was typical. It causes an irregular distribution of genetic material among daughter organelles, renewal of division of mature cells, increases in the heterogeneity and heteromorphism of cells, their ability for selective response, and at the same time deformities of leaves and plants in the aggregate.

SENSITIVITY OF PLANT CYTOGENETIC AND
GENETIC SHORT-TERM ASSAYS FOR EVALUATING
GENETIC DAMAGE INDUCED BY CHEMICAL
MUTAGENS

P. Kuglik, R. Veselská, J. Relichová

Department of Genetics and Molecular Biology, Faculty of Sciences, Masaryk University, Kotlářská 2, 611 37, Brno, Czech Republic

A number of assays has been developed which use higher plants for the detection of environmental agents with mutagenic potential.

In our experiments, the level of the sensitivity of plant cytogenetic assay (detection of sister chromatid exchanges in *Vicia faba*) was compared with plant genetic assays (Muller's embryo test for gametic mutations in *Arabidopsis thaliana*, *Tradescantia* stamen hair mutation test) used as the most favorable preliminary screening tests for monitoring of environmental mutagens. As model chemical mutagens, ethylmethanesulphonate (10^{-2} - 10^{-4} M), N-methyl-N-nitrosourea (10^{-4} - 10^{-7} M) and maleic hydrazide (10^{-5} - 10^{-6} M) were used.

The results showed that *Tradescantia* stamen hair assay was the most sensitive plant system for the testing of mutagenic effect of alkylating agents. In the case of maleic hydrazide, cytogenetic analysis of sister chromatid exchanges in *Vicia faba* cells was proved to be the most effective. It was found that cytogenetic detection of sister chromatid exchanges in *Vicia faba* cells was equally or more sensitive than *Arabidopsis thaliana* assay.

These results are another indications that both plant cytogenetic and genetic assays may be useful as effective monitors of environmental mutagens.

Th-22 OBTAINING INTERSPECIFIC HYBRIDS
BETWEEN FAGOPYRUM ESCULENTUM
AND FAGOPYRUM TATARICUM VIA EMBRYO
CULTURE

M. Rubtsova, B. Levenko, L. Tarantenko, A. Shapoval.

Institute of Plant Physiology & Genetics, Ukrainian Acad. Science, Kiev, Institute of Plant Production, Ukrainian Acad. Agric. Science, Kiev, Ukraine

Wild species of buckwheat are known as donors of various valuable characters: heat, drought, chilling, lodging tolerance, self-fertility. Unfortunately it is impossible to obtain interspecific hybrids of buckwheat using routine methods, due to interspecific incompatibility. Using immature embryo culture interspecific hybrids between *F.esculentum* and *F.tataricum* ssp. *rotundatum* have been obtained.

The reliable method of the selection of hybrid plants was worked out. Time of pollination and embryo isolation and conditions of embryo culture were investigated. Best adaptation to the *in vitro* conditions was achieved for 12-14 day embryos. Concentrations of auxins and cytokinins, that stimulate shoot and root formation of hybrid plants were investigated. Hybrid plants normally developed, formed roots and flowered.

263 hybrid plants were selected using these procedures. 10 cm plants were brought to a greenhouse for acclimatization and then to the field. The genuine hybrid nature of these plants was confirmed by the isoenzyme analysis.

TRANSGENIC TOBACCO PLANTS WITH
FISSION YEAST *cdc25* UNDER AN
INDUCIBLE PROMOTER

Th-24 R. McKibbin^a, D. Francis^a and N.G. Halford^b, ^a School of Pure and Applied Biology, University of Wales, Cardiff and ^b Institute of Arable Crop Research, Long Ashton Research Station, University of Bristol, UK.

We are studying the effects of the *S. pombe* gene, *cdc25*, (a mitotic inducer) on cell division in transgenic tobacco plants. *cdc25*, under the control of a tetracycline-inducible promoter, was integrated into the tobacco genome using Agrobacterium-mediated transformation. The presence of the gene was detected by Southern blots.

Roots from transgenic plantlets were cultured in a modified Murashige-Skoog liquid medium in darkness at 27°C. Tetracycline at 1 mg l⁻¹ was added to the medium at 3 day intervals and roots were fixed at 7 day intervals following the start of culture. The rate of secondary root formation, the size of lateral root primordia and cell size in the primordia were measured in the transgenic and control roots + tetracycline. Preliminary data indicate that the mean size of lateral root primordia was significantly smaller in the transgenic roots in the presence of tetracycline.

EPIGENETIC MODIFICATION OF PLANT NUCLEAR GENOME

Th-25 B. Vyskot

Institute of Biophysics, Academy of Sciences, 612 65 Brno, Czech Republic

Some epigenetic phenomena extensively studied in higher animals have been recently described in plants, too. They include parental imprinting in endosperm, paramutations, transgene silencing, functional diploidization in polyploids, and parental dominance in hybrids. In some cases it has been unambiguously proved that gene inactivation is correlated with DNA methylation. We present data indicating that one X chromosome hypermethylation and late replication, as dosage compensation mechanisms described in mammals, can occur in homogametic dioecious plant *Melandrium album*. DNA methylation patterns are controlled by developmental processes, but they can be modified by various environmental stress factors (e.g. in vitro culture conditions) or hypomethylating drugs. Using 5-azacytidine, even highly repetitive DNA sequences can be demethylated and this status is inherited through many mitotic divisions. Here we demonstrate its ability as a tool to study developmental pathways in plants. In *Nicotiana tabacum*, a 5-azacytidine treatment regularly leads to floral changes strikingly resembling homeotic mutations. In dioecious species *M. album*, the same treatment induces a sex reversal, hermaphrodite flowers on male genotypes, apparently by an inhibition of the Y chromosome provided suppression of female sex genes.

NUCLEAR CHANGES IN HETEROPHYLLOUS
TRAPA NATANS BUDS

Th-26

Cozza R., Ruffini-Castiglione M.* S. Mazzuca, M. B. Bitonti and A. M. Innocenti.

Dipartimento di Ecologia, Università della Calabria, 87030 Arcavacata di Rende (CS), Italy; * Dipartimento di Scienze Botaniche, Università di Pisa, Via L.Ghini 5, 56100 Pisa, Italy

Trapa natans (Trapaceae) is an annual floating-leaved plant confined to northern Italian lakes, which during its vegetative development produces submerged and floating leaves with heteromorphic characteristic. In order to furnish some interesting insights concerning the relationship between morphogenetic development model and nuclear chromatin organization in *Trapa natans* buds, morphometric and cytochemical analyses were carried out on meristematic nuclei in buds and leaf primordia developing in the two kind of leaves. As for the chromatin structure, at the light microscopy the nuclei of both buds (S=submerged; F=floating) display an articulate organization, with prominent chromocentres showing a peripheral arrangement beneath the nuclear envelope. When the nuclear size of meristematic cells is considered, F buds nuclei display a higher nuclear and nucleolar area and a lower number of chromocentres than S buds. These differences are associated with nuclear cytochemical changes in meristematic cell populations of the two kind of buds. On the basis of DNA methylation being related to both gene expression and chromatin structure, we also tested using immunocytochemical techniques the level of DNA methylation in these two nuclear populations. The localization of antibody against 5-methyl-cytidine in shoot apex nuclei reveals important quantitative differences between the two kind of buds. Infact the level of immuno-binding is clearly higher in F bud meristems which display strongly labelled nuclei respect to the weakly labelled nuclei of S bud. The results are discussed in relation to possible mechanisms underlying heterophyly phenomenon.

THE MAIZE MITOCHONDRIAL GENE
T-*URF13* INFLUENCES FLOWER

Th-27 DEVELOPMENT IN TRANSGENIC TOBACCO

C. Hartung, L. Borchert, W.O. Abel &
R. Lührs

Institute of Botany, Department of Genetics, Ohnhorststraße 18, D-22609 Hamburg, Federal Rep. of Germany

Tobacco plants were transformed via *Agrobacterium tumefaciens* with the maize mitochondrial gene T-*urf13*. In maize lines with Texas cytoplasm the expression of this chimeric gene is associated with cytoplasmic male sterility. For targeting the gene product to tobacco mitochondria the T-*urf13* gene was fused to the *Nicotiana plumbaginifolia* presequence of the β-subunit of the mitochondrial ATPase under control of the l' promoter.

Twenty transgenic tobacco plants were already transferred to soil: 50% of the plants did not show any differences compared to untransformed controls, but flower morphology of the remaining 50% were influenced by the T-*urf13* gene. Generally, flower and pollen development was delayed. Flower colour was more pale and the length of the filaments were reduced preventing spontaneous pollination. Two plants were sterile, they showed abnormal stamens, pollen number and viability were extremely low.

RESEARCH OPPORTUNITIES ASSOCIATED
WITH GENETIC MODIFICATIONS OF TOMATO
PLANTS AGAINST TOMATO YELLOW MOSAIC
VIRUS (TYMV)

Th-28

N. Piven^{a,b}, V. Rudas^b, D. Infante^a.

^a. Instituto Venezolano de Investigaciones Científicas, Apdo. 21827, Caracas 1020-A, Venezuela.

^b. Institute of Cell Biology and Genetic Engineering Zabolotnogo Str., 148, 252022, Kiev, Ukraine.

The aim of this research is the improvement of genetic resistance to TYMV in tomato. After testing different accessions of wild species of *Lycopersicon*, *L. chilense* and *L. peruvianum* v. *dentatum* were found to be highly resistant and tolerant to TYMV. Transgenic plants of *L. peruvianum* v. *dentatum* and interspecies hybrids between *L. chilense* and *L. esculentum* were obtained and tested to the TYMV. Reduced susceptibility to the TYMV disease were observed. The results showed some possibilities to increase resistance to TYMV by different ways.

Th-29 ANATOMICAL STUDY OF SOMATIC EMBRYOGENESIS IN GRAIN LEGUMES
(Pisum sativum L., Vicia faba L., and Glycine max (L.) Merr.)

M. Griga

OSEVA - Research Institute of Technical Crops and Legumes, 787 01 Šumperk, Czech Republic

The anatomy of initiation, development and conversion of somatic embryos in three economically important grain legume crops - pea, faba bean, and soybean - was studied. Somatic embryos were induced either directly from initial explants (direct somatic embryogenesis) or in callus culture (indirect somatic embryogenesis). In addition, somatic embryos from long-term embryogenic cultures (repetitive embryogenesis) were analysed. A number of auxins (2,4-dichlorophenoxyacetic acid, α -naphthaleneacetic acid, picloram, dicamba) were successfully tested as inducers of somatic embryogenesis from whole immature zygotic embryos, embryo axes, cotyledons and shoot apices of aseptically germinated seedlings. The stress was laid on localization of initiation sites of somatic embryogenesis in the tissues of initial explants and calli. The morphological and anatomical similarities and differences of somatic and zygotic embryos were discussed.

Th-31 EXTRACELLULAR MATRIX IN EARLY STAGES OF PLANT REGENERATION IN VITRO

J. Šamaj^a, M. Bobák^a, M. Oyečka^b, J. Kristín^a and A. Blehová^a

^aDepartment of Plant Physiology, Comenius University Bratislava, Mlynská dolina 8-2, 842 15 Bratislava, Slovak Republic

^bDepartment of Pharmacology and Toxicology, Comenius University Bratislava, Kalinčiakova 8, 832 32 Bratislava, Slovak Republic

Network and band-like extracellular material was observed on the surface proembryoid cells of *Papaver somniferum* L. Single proembryoid cells were connected by network, while band material was present mostly between proembryoids. Moreover, small globular separated cells with needle-like extracellular matrix were also present on the surface of embryogenic *Papaver* tissue. Continuity between structure of needle-like material and cell shape may indicate its functional connection to cytoskeleton.

Fibrillar network representing an extracellular matrix surrounding proembryoids was also present during early regeneration in leaves of *Drosera rotundifolia* L. The network was gradually degraded to coarse strands of fibrils between neighbour cells during proembryoid differentiation.

Extracellular matrix disappeared while protodermis was formed in globular shaped embryos. The presence of the extracellular matrix can serve as an early structural marker in plant regeneration.

CHOLINESTERASE IN POLLEN
Th-30 V.V.Roshchina^a, E.V.Melnikova^a, N.V.Spiridonov^b, L.V.Kovaleva^c, N.N.Semenova^d
^aInst.of Cell Biophysics; ^bInst.of Theor.and Exper.Biophysics,Pushchino,
 142292; ^cInst.of Plant Physiology; ^dInst.of Developmental Biology, Moscow, Russia.

Cholinesterase activity, measured as hydrolysis of acetylthiocholine (ATCH) or butyrylthiocholine (BUTCH) by histochemical and biochemical methods, was found in 8 species from 14 studied natural pollen and 2 types of pollen loads bee-collected. The ATCH hydrolysis was inhibited by the animal cholinesterase inhibitors neostigmine and physostigmine ($K_1=10^2-5\times 10^3\text{ M}^{-1}$) $\% \text{ of inhibition}$ varied from 20 up 100. The phenomenon was observed in *Philadelphus multiflorum*, *Fetunia hybrida*, *Hippeastrum* sp., *Salix caprea*, *Papaver orientale*, *Matricaria chamomilla*, *Pinus sylvestris*, but not in *Betula verrucosa*, *Plantago major*, *Geranium*, *Nelvis domestica*, *Populus balsamifera*. The cholinesterase of native pollen has no inhibition by substrate, but the specificity to ATCH was ~3 fold higher than to BUTCH. Pollen loads have 10 fold lower activity of the enzyme than native pollen. The presence of cholinesterase activity in male cells of plants may be connected with the participation in fertilization.

Th-32 FINE STRUCTURE OF RYEGRASS GLOBULAR SOMATIC EMBRYOS

Pavlova M.K., Podlutsky A.G.

Department of Cell Biology and Anatomy
 Institute of Botany, Acad. Sci., Kiev, Ukraine

Embryogenic tissue cultures of ryegrass (*Lolium multiflorum*) were obtained from shoot apexes on Murashige and Skoog medium (MS) supplemented with 5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 100 ml/l coconut water. Nodular calli have formed with 3 weeks of culture. Somatic embryogenesis was observed in cell suspension cultures initiated from calli cultured in MS liquid medium supplemented with 0.5 mg/l picloram, 0.5 mg/l 2,4-D, 20 mg/l silver nitrate. Macroscopically visible small globular embryos were organized in 4-week-old cultures. More detailed studies have shown that these embryos were embedded in groups of 3 or 5 ones. The electron microscopic studies have shown that toward the center of globula cells are small, thin-walled, non-vacuolated, with prominent nuclei and rich cytoplasm. Some of cells have contained amyloplasts with prominent starch grains. We have observed the group of cells (3-4) with the crystalline inclusions in the plastids. These inclusions are similar to phytoferritin aggregates. The data obtained are discussed regarding to cellular interactions during the formation of embryos in plant tissue culture.

Th-33CALLUS TYPES AND ORGANOGENESIS IN SOME
CACTACEAE SPECIES

M. Corneanu¹, G.C. Corneanu¹, C. Crăciun²,
V. Crăciun²

¹Genetics Sect., Craiova University, 1100 -
Craiova; ²Experimental Biology Dept., Babeş - Bolyai
University, 3400 - Cluj, Romania.

The callus structure and organogenesis processes in *Mammillaria duwei* Rog. et Brown, *M. flavescens* (DC) Haw and *Coryphanta elephantides* (Lem.) Lem. was studied. There are two different callus types (friable and compact) with different structure and features. Friable callus is composed from cells clusters, superficial bind between them, with features depending on genotype. In *M. duwei* there are 3 different cells types: young cells, rich in cytoplasm, grouped in clusters; spherical cells, mature, very big; cylindrical cells, grouped in rows. In these cells is present a single nucleolus. In *M. flavescens*, the callus is formed by a single cells type, spherical, with three nucleolus. *C. elephantides* present two cells types: young cells with unregulated outline, big, rich cytoplasm with numerous lipidic drops and mature cells, big, perfect spherical with few cytoplasm and a single nucleolus.

The organogenesis process is present only in the compact callus. In *M. duwei* this present at external surface an unstratified epidermis. Assimilator parenchyma is formed of big cells with pellicular cytoplasm and cellular organelles disposed at the cell periphery, with structure dependent on genotype and callus region. They present a big quantity of liquid. The chloroplasts present few thylakoids, unorganized in grana, rarely small plastoglobuls, starch grains and sometimes paracrystalline structures. The nucleolus present small heterochromatin blocks at its periphery. Organogenic region present the look of one excrescence on the callus surface. The long walls of the middle formed foldings. Through their thicken, they will be transformed in ligneous cells with partial sclerified lateral walls (under form of some spirals). They are surrounded by long cells which will be transformed in the liberian vessels.

Th-35CLONAL MULTIPLICATION IN FOREST TREES
BY MEANS OF ORGANOGENESIS AND SOMATIC
EMBRYOGENESIS

Jana Malá, P. Šíma

Forestry and Game Management Research Institute
Jíloviště - Strnady, Prague 5, Czech Republic

Explants taken from the shoots and seeds of *Quercus petraea*, *Fagus sylvatica*, and *Ulmus laevis* were cultured in vitro in solid agar medium containing nutrient media with various combinations and/or concentrations of auxin, cytokinin, glutamate and casein hydrolysate. After primary explants induction the plant material was transferred into the flasks containing media supporting development of somatic embryos or growth of axillary buds. The various developmental stages of somatic embryos and new growing shoots were used for conversion or rooting. After selection of optimal cultivation conditions the viable population suitable for propagation has been established in all broad-leaved trees.

Th-34HISTOLOGY AND MORPHOLOGY OF
GLADIOLUS SOMATIC EMBRYOS

B.Stefaniak

Department of General Botany,
Faculty of Biology,
A.Mickiewicz University,
Poznań, POLAND

Regeneration of plants via somatic embryogenesis is considered to be an efficient method for clonal propagation.

Friable embryogenic callus and somatic embryos of four Gladiolus hort. cultivars were obtained on solid MS medium supplemented with auxins, using corm slices, young leaf bases and whole intact plantlets as explants. Somatic embryos transferred on hormone-free medium regenerated into plants. From friable callus of all tested cultivars embryogenic cell suspension cultures were established. The cell suspensions were composed of small aggregates and dissociated single cells. When cell suspensions were transferred on solid medium they formed friable callus and then somatic embryos. Later on, these somatic embryos developed into green plants.

Embryogenic callus and cell suspension have been maintained in culture for over 2 years and they still retain very high embryogenic capacity.

The histology and morphology of the obtained somatic embryos were examined.

Th-36*In Vitro* ORGANOGENESIS AND PROTOPLAST
CULTIVATION OF SUGARBEET (*Beta vulgaris*
L.)

Bannikova M. A., Golovko A.E., Khvedynich O. A.,
Kuchuk N.V., Gleba Y.Y.

Institute of Cell Biology & Genetic Engineering, Zabolotnogo str.,
148. Kiev 252022, Ukraine

Aseptic sugarbeet plants of Ukrainian commercial lines were grown on MS hormone free nutritional medium. Petioles, hypocotyls, leaves, basal tissue were used as explants. The shoot regeneration was obtained on MS medium supplemented with 1 mg/l BAP. The regeneration frequency could rise up to 75% while petiole with leaf was taken as explant. In this case the regeneration processes happened at petiole's surface along the axle fibre. The sugarbeet regeneration process studied by histological analysis was performed as organogenesis. Most successfully process of callus formation occurred on the MS hormone free medium while petiole's regenerants were used as explant. The obtained callus were cultivated on MS medium supplemented with 0.25 mg/l BAP. High regeneration ability of callus lines was noted. Protoplasts were isolated from callus sugarbeet lines. Protoplast divisions has been detected after 5-7 days of cultivation in KM liquid medium. Microcolonies have been formed after one month of cultivation and were placed in KM medium with 1/2 osmotic pressure. While callus colonies have reached 1.5 - 2 mm they were removed to MS agar with 1mg/l BAP. At present, shoot induction experiments from the protoplast derived callus are being performed.

Th-37 Havel L., Durzan D.J.
 University of Agriculture, Dept. Botany,
 Zemědělská 1, 613 00 Brno, Czech Republic,
 University of California, Dept. Environmental
 Horticulture, 95616 Davis CA, USA

The somatic embryos in conifers are longitudinal structures comprised of an embryonal group of cells (E), embryonal tubes (ETs) and an embryonal suspensor (ES). The E is composed of isodiametric cells with high nucleus to cell ratio. The ETs arise from the E to initiate the formation of the axial tier. ETs extend in one direction very fast with a low nucleus to cell volume ratio. The nucleus may be larger than in E cells. This region of the early embryo maintains tight intercellular connections in both radial and longitudinal directions. Cells at the distal end of the ET tier gradually loose their radial connections to start the differentiation of the ES. These predictable process involves the degradation of the nucleus in ET cells and the extrusion of nucleoli into the cytoplasm. At the distal end of the ES region, the cells collapse and their debries are released into the culture medium. All aspects of the differentiation of the axial tier are easily supported cytochemically by a variety of stains. Results comprise a new model for the ontogeny of the early embryo.

Th-39 INCREASE OF SOMATIC EMBRYOGENESIS AND SHOOT MORPHOGENESIS IN MAIZE INBRED CALLUS CULTURES
 T. Checheneva, V. Trukhanov

Institute of Plant Physiology and Genetics,
 Kiev, Ukraine

Maize plant regeneration in vitro may be achieved by means of shoot morphogenesis /I type/ or somatic embryogenesis /II type/ from totipotent callus cultures. Many of valuable inbreds form with high frequency nontotipotent callus which posseses regeneration ability during a short period of time. Pioneer 502, Pioneer 346 and BS 2923 used in experiments may be characterized so. Significant increasing of somatic embryogenesis frequency was obtained on regeneration medium added by mannitol. Intensive somatic embryo germination was initiated by sub-cultivation with BAP during one passage. AgNO₃ containing medium prolonged a regenerant formation period from three to eight months.

All used methods give an opportunity to widen a maize inbred line collection for biotechnological manipulations.

Th-38 CYTO-EMBRYOLOGICAL STUDIES OF LOTUS CORNICULATUS L. (FABACEAE)

M.Chubirko

Uzhgorod State University, Dept. of Botany, Uzhgorod 294000, Ukraine

The anthers of *Lotus corniculatus* L. are bilobed and tetransporangiate. In early stage development the hypodermal cells of anther divide mitotically in random planes. Eventually the anther becomes four-lobed, each lobe consists of sporogenous cells bounded by the tapetum, the middle layer, the endothecium and the epidermis.

The divisions of the sporogenous cells cease and some increase in cell size occurs. Microporesocytes enter the prophase I of the division meiotic. They complete first and second division. Simultaneous cytokinesis in the microporesocytes follows meiosis.

The ovules are bitegmic crassinucellate anatropous. They are formed in monocarpely ovary. The archesporium in the ovule is hypodermal, cuts off a parietal cell and forms megasporocytes. Meiosis I and transverse division of this cell lead to a dyad stage. While the lower cell of the dyad invariably divides transversely, the division in the upper cell may either be transverse or longitudinal resulting in linear or T-shaped tetrads. The chalazal or epicchalazal megasporite is the largest one of the four and it divides mitotically to form the female gametophyte which consists of an egg, two synergids, two polar nuclei and three antipodal cells. The development of the embryo sac conform to the *Polygonum* type.

Th-40 STUDY OF THE COMPOSITION OF EXTRACELLULAR POLYPEPTIDES DURING SOMATIC EMBRYOGENESIS IN *MEDICAGO TRUNCATULA*

M.L. Couto^a, A.S. Lopes^a, J. Costa^a, M.P. Fevereiro^{a,b}

^aI.T.Q.B., U.N.L., Apartado 127, 2780 OEIRAS; ^bDepartamento de Biologia Vegetal, F.C.U.L., Ed. C2, 1700 LISBOA - PORTUGAL

The identification of molecular markers during somatic embryogenesis may constitute a basis for further studies of the differentiation process.

Induction of somatic embryogenesis in suspension cell cultures of *M. truncatula* occurred in B₅ medium supplemented with 50 µM of 2,4-dichlorophenoxyacetic acid, either in the presence or in the absence of 5 µM kinetin. Embryo maturation was obtained after change to modified B₅, hormone-free medium. Somatic embryos differentiated in this medium have gone through distinct morphological stages: globular, heart, torpedo and cotyledonary.

The extracellular polypeptides from *M. truncatula* cell cultures were analysed by SDS-PAGE, during induction (0, 5 and 12 days) and maturation (0 to 6 weeks) phases. Most remarkable was the presence of a 46 kDa polypeptide, whose concentration increased during the induction phase and decreased during the maturation phase, both in the presence and in the absence of kinetin. This polypeptide was found to be glycosylated, having affinity for concanavalin A.

The 46 kDa glycosylated polypeptide might be related to the differentiation of somatic embryos from *M. truncatula*.

Th-41 ORIENTATION OF CELL DIVISION DURING PREGLOBULAR PHASE OF POLLEN EMBRYOGENESIS IN RAPESEED MICRO-SPORE CULTURES

Z.Opatrný, M.Vyvadilová

Research Institute of Crop Production,
Drnovská 507, 170 00 Prague, CR

The development of microspores in "pollen" cultures of rapeseed (spring or winter varieties of *Brassica napus*) proceeded only in two ways - either toward differentiation of mature, non-germinating pollen grains or through stepwise formation of pollen embryos. Indirect (*via callus*) embryogenesis was never observed, no callogenesis could be induced even by various hormonal treatment. The first cell division of microspores was either equal or unequal, with comparable frequency and in both cases focusing to embryogenesis. Consequently, the handed down importance of symmetry/asymmetry of the cell division for the determination of following development of descendants was not proved. The subsequent cell division was mostly chaotic, pronouncedly disoriented. Only after multicellular (30-40) mass formation during 5-6 days of culture, the distinct core layer, consisting of small uniform cells differentiated, probably as necessary prerequisite of further development of globular to heart-shape and torpedo embryos.

Th-43 ZYGOTIC EMBRYOGENESIS IN VITRO FROM FUSED ISOLATED GAMETES OF MAIZE

E.Kranz and H. Lötz

Institute of General Botany, University of Hamburg, Ohnhorststrasse 18, D-22609 Hamburg / Germany

In vitro fertilization with single isolated egg and sperm cell protoplasts results in zygotic embryogenesis and phenotypically normal, fertile maize plants (Kranz and Lötz, 1993, Plant Cell 5: 739-746). The electrofusion of the gametic protoplasts leads to the efficient formation of polar zygotes, globular structures, proembryos and transition-phase embryos and to the formation of hybrid plants from individually cultured fusion products. The early stages of embryo development created in vitro strongly resemble those of a maize plant after natural fertilization. High-frequency karyogamy during in vitro fertilization was demonstrated (Faure et al., 1993, Plant Cell 5: 747-755).

We also present evidence for the fusion of isolated single maize sperm and egg cell protoplasts by high calcium and high pH, followed by cell division of the fusion products (Kranz and Lötz, 1994, Zygote, in press). The relevance of these findings to future developments in adhesion, recognition and fusion research of higher plant gametes will be discussed.

Prospects of the possibility to fuse two gametes of the opposite sex combined with individual culture and growth of the zygote in vitro in the study of fertilization processes and experimental embryology are also discussed.

IMAGING CALCIUM DYNAMICS IN LIVING PLANT CELLS AND TISSUES

N.D. Read

Molecular Signalling Group, Institute of Cell and Molecular Biology, University of Edinburgh, Rutherford Building, Edinburgh EH9 3JH, United Kingdom

Eukariotic cells have fashioned a complex network of signalling capabilities around Ca²⁺. The central role of Ca²⁺ signalling in plants is now well established. Much of the work has concentrated on imaging free Ca²⁺ in living plants using fluorescent dyes (confocal microscopy and fluorescent ratio imaging) and recombinant aequorin (photon counting imaging). Recent results obtained using these techniques will be described, with particular emphasis being paid to our studies on pollen tubes in plants.

CONNECTION BETWEEN HEAT SHOCK PROTEINS AND CELL PROLIFERATION

Th-44 Paul M. Pechan

Max Planck Institut für Biochemie,
82152 Martinsried, Germany

Stress elicits response in affected cells. The cell response may take a number of forms. In some organisms, for example plants, this leads to the induction of cell proliferation. A number of treatments, including high temperatures, osmotic shock and hormonal starvation are used to initiate cell proliferation in plant cultures. As all known stress response mechanisms include the synthesis of heat shock proteins (Hsps), an important question to ask is what role these proteins could play in inducing cell proliferation (Pechan, FEBS 280:1-4 1991). There is growing evidence that some constitutively expressed Hsps can be involved in cell proliferation under non-stress conditions. It is proposed cells which do not synthesize Hsps in a developmentally regulated manner, could under certain conditions undergo cell proliferation process when Hsps appear in cells in response to stress. Interesting correlations can be drawn between stress, Hsps, cell proliferation in plants and cancer.

PLANT EMBRYOGENESIS-REVIEW AND PROSPECTS

Th-45 J.M.Dunwell

ZENECA Seeds, Jealott's Hill Research Station, Bracknell, Berkshire, TG12 6EY United Kingdom

The process of embryogenesis, whereby a single cell zygote undergoes a complex sequence of cell divisions and differentiation to produce a bipolar structure capable of germination, has been the subject of much investigation over the decades. In the original studies, most of the focus was on anatomical and histochemical description but more recently two significant advances have been made. Firstly, it is possible, under in vitro conditions, to recapitulate the process of embryogenesis from a single cell, being it zygotic embryogenesis (e.g. maize), a somatic cell (e.g. carrot) or an immature gametophyte (microspore) of species such as tobacco or barley. Secondly, progress in molecular biology and genetics has allowed both a detailed analysis of gene expression during the different stages of embryo development, and the induction of mutants known to be defective at known stages in this process. This review will consider these recent advances and place them in the context of general plant biology.

CANCELLED

ISOLATION OF A GENE CORRESPONDING TO A PHOSPHOPROTEIN SPECIFIC TO EMBRYOGENIC POLLEN IN TOBACCO.

M. Kyo

Faculty of Agriculture, Kagawa University, Kagawa 761-07, Japan

We have previously established pollen culture systems for observing early events of pollen embryogenesis in *N.tabacum* (Planta 168: 427-432, 1986) and in *N.rustica* (Plant Physiology 79: 90-94, 1995). 2-D electrophoretic patterns of total phosphoproteins isolated from in vitro cultured pollen grains indicated that several phosphoproteins (EPPs) appear in embryogenic pollen of *N.tabacum* (Planta 182: 58-63, 1990) and also in *N.rustica* within 3 days of culture initiation. These EPPs were partially purified and transferred onto PVDF membrane for analysis of their N-terminal amino acid sequences. The analysis revealed all of the EPPs in *N.tabacum* and *N.rustica* possess a similar sequence in their N-terminal. Using synthesized oligo DNA fragments from the N-terminal amino acids as probes it was possible to isolate a gene from the cDNA library which encoded the N-terminal region of one EPP. The protein sequence of this gene showed approximate 30% homology to phosphorylase, nodulin and stellacyanin proteins.

Th-48 EXPRESSION OF FIBRONECTIN IN HUMAN AND PORCINE FOETUSES

I. Trebichavský^a, O. Nyklíček^b

^a Department of Immunology and Gnotobiology Institute of Microbiology, Prague; ^b Department of Gynaecology and Obstetrics, Hospital Náhod, Czech Republic

Fibronectin is a family of closely related glycoproteins of extracellular matrix. It plays multiple roles in differentiation, wound repair, cell adhesion, migration and proliferation. In prenatal development, fibronectin has been shown to be deposited in specific sites and to regulate the migration of embryonic cells. Using immunofluorescence and avidin/biotin method, we investigated the distribution of fibronectin in human foetus (14 gestational weeks) and in pig foetuses (29, 40, 43, 50, 73, 80 and 90 days of gestation). Fibronectin was strongly expressed in intestines, lungs, heart and skin of both species. Stromal cells of immune organs - splenic reticulum and thymic epithelium expressed the fibronectin in distinct period of development.

PHAGOCYTIC ACTIVITY AND LECTIN BINDING PROPERTIES OF MOLLUSCAN HAEMOCYTES.*

Th-49

M.P. Cajaraville, Y. Robledo, I. Olabarrieta, I. Mariñómez, J.F. Madrid, J. Ballesta (a) and S.G. Pal. Biología Zelularra eta Zientzia Morfológiako Saila, UPV/EHU, Bilbo; (a) Dto Biol Celular, Univ. Murcia.

Due to the open circulatory system of molluscs, blood cells or haemocytes are found in the connective tissues throughout the animal and in the circulating haemolymph. Haemocytes constitute the main internal defense system in molluscs, which is brought about mainly by phagocytosis and cell-mediated cytotoxicity. Lectins are known to be produced by molluscan haemocytes and seem to be decisive for phagocytosis by acting as recognition factors. In the present study we investigated the uptake of latex beads and the dyes neutral red (NR) and acridine orange (AO) by isolated spread haemocytes of *Mytilus galloprovincialis*. Only some of the granules of haemocytes are stained with NR and AO. This functional heterogeneity of the granules was demonstrated by acid phosphatase cytochemistry, too. In addition, fusion between granules and phagosomes was demonstrated by TEM. We also applied lectin histochemistry to investigate cell surface carbohydrates of the haemocytes on digestive gland tissue slices stained with a battery of 16 HRP- or digoxigenin-labeled lectins. Haemocytes found both within haemolymph spaces and in tissues were reactive to WGA, DSA, Con A, GNA, PHA-L and AAA. No reactivity was observed for LFA, BSI-B4, MPA, PNA, DBA, MAA, SNA, UEA I and LTA suggesting the lack of galactose, sialic acid, N-acetyl galactosamine and fucose residues linked to galactose in haemocytes. The labeling of HPA was variable and non uniform among the haemocytes; this is probably due to the existence of different haemocyte subpopulations. In summary, in mussel haemocytes N-acetyl glucosamine (GlcNAc), mannose and fucose residues are present. The latter are selectively located at a terminal position or linked to the innermost GlcNAc residues of N-linked glycoproteins of the plasma membrane. These cell surface glycoconjugates may play prominent roles in intercellular communication, cell adhesion and recognition in relation with phagocytosis of foreign bodies.

* Funded by CICYT (AMB 93-0432) and DGICYT (SAB 93-0170).

$\alpha\beta 1$ INTEGRIN MEDIATES FORMATION OF NORMAL BREAST ACINUS-LIKE SPHERES BUT NOT BREAST TUMOR COLONIES WITHIN RECONSTITUTED BASEMENT MEMBRANE

Th-51 Anthony R. Howlett^a, Nina Bailey^a, Caroline Damsky^b, Ole W. Petersen^c & Mina J. Bissell^a. ^aLawrence Berkeley Laboratory, UC Berkeley, CA 94720 USA; ^bDepts of Stomatology and Anatomy, UC San Francisco, CA 94143 USA; ^cPanum Inst. University of Copenhagen, Denmark.

We previously developed an assay for the study of human breast cancer where normal breast epithelial cells and breast tumor cells recapitulate many aspects of their *in vivo* patterns of growth and differentiation when placed within a laminin rich matrix of reconstituted basement membrane. We used this assay to study the expression and function of potential laminin receptors in normal breast epithelial cells and their tumorigenic counterparts. We show by immunohistochemistry, that normal cells weakly express $\alpha 1$ subunits in the cytoplasm and $\alpha 2$ at the basolateral surface while $\alpha 3$ and $\beta 1$ subunits are abundantly expressed at the basolateral surface and $\alpha 6$ and $\beta 4$ at the basal surface. These data suggest pairing of $\alpha 3$ with $\beta 1$, $\alpha 6$ with $\beta 4$ and potential pairing of $\alpha 2$ and $\alpha 6$ with $\beta 1$. In contrast to normal cells, three tumor cell lines showed loss or downregulation of the same integrin subunits. Metastatic MDA-MB435 breast carcinoma cells showed loss of the $\alpha 2$, $\alpha 6$ and $\beta 4$ subunits and downregulation of $\alpha 3$ and $\beta 1$ while the non metastatic lines HMT 3909/S13 and MCF-7/9 showed disordered expression and/or losses of the same subunits. Subsequent function blocking experiments using anti- $\beta 1$, $\alpha 2$, $\alpha 3$ and $\alpha 6$ antibodies showed a greater than 5 fold inhibition of the formation of organotypic spheres by normal cells in the presence of either anti- $\alpha 3$ or anti- $\beta 1$ antibodies but no significant effect was observed for anti- $\alpha 2$ or $\alpha 6$. The inhibitory antibodies had no effect on colony formation by tumor cells. These data suggest that the $\alpha 3\beta 1$ integrin is a functional receptor for signals regulating acinus-like sphere morphogenesis in breast epithelial cells in response to basement membrane. This pathway appears to be down regulated or lost in breast tumor cells suggesting that tumor colony formation occurs by independent mechanisms and that abnormalities of integrin mediated cell-ECM interaction may be critical to breast tumor formation.

TNF α Interacts with Fibronectin; Effects on Immune Cell Activation and Adhesion

Th-50

L. Cahalon^a, T. Sapir^a, R. Hershkoviz^a, S.K. Akiyama^b, K.M. Yamada^b, and O. Lider^a

^aThe Dept. of Cell Biology, The Weizmann Institute of Science, Rehovot, Israel, and ^bNIDR, NIH, Bethesda, Maryland, U.S.A.

We have examined the association of TNF α with glycoprotein constituents of ECM. TNF α interacted with immobilized or soluble fibronectin (FN) and laminin. The major binding site for TNF α on FN was localized to its 30-kDa N-terminal fragment (FN-N') with a Ki in the nM range. The effects of TNF α on immune cells may be influenced by their milieu; FN-bound TNF α augments the level of adhesion of activated CD4 $^+$ cells; a brief exposure of CD4 $^+$ cells to low doses of soluble or of FN- or LN-bound TNF α , synergized with PMA to enhance the integrin-mediated binding of CD4 $^+$ cells to these immobilized ECM moieties. When TNF α was immobilized on FN, less TNF α was required to induce CD4 $^+$ cell binding to FN. Soluble, and to a greater extent FN-bound TNF α , synergizes with PMA to intensify protein tyrosine-phosphorylation in FN-bound CD4 $^+$ cells. Interestingly, FN-bound or soluble TNF α also amplified the binding of an Ag-specific autoimmune rat T cell line to FN. This emphasizes the physiological relevance of our findings. Thus, the cell-adhesive properties of ECM-glycoproteins may be modulated by their association with TNF α , and matrix-linked TNF α may recruit and direct immune cells to inflammatory sites.

PLANT POLYSACCHARIDES CAN SUPPRESS GROWTH OF NORMAL AND TRANSFORMED FIBROBLASTS IN CULTURE

Ivan A. Diakonov, Yu.V. Gorelik, M.I. Blinova, L.V. Kukhareva.

Institute of Cytology, Sci. Acad. Russia, Tikhoretsky av 4, 194064 StPetersburg, Russia

Recently heparan-like animal polysaccharides (PS) were shown to suppress growth of smooth muscle and hepatoma cells. (For review see: Jackson R.L. et al, 1991, Physiol J.v.71, p. 481). In the present work we checked plant PS as growth inhibitors in animal tissue culture system. In the growth-arrested and restimulated fibroblasts some PS such as heparin, dextran sulfate, and alginate blocked the stimulation of ^{3}H -thymidine incorporation, while another sulfated PS, rodexman sulfate, did not. Since we found exactly the same effect in serum-free cultures stimulated with pure epidermal growth factor which have not been shown to bind to PS, it seems likely that PS effect is not related to its ability to bind proteins such as growth factors.

As for normal fibroblasts, for transformed cell lines we observed growth inhibition. The plating efficiency was as little as half of control in the presence of alginate, carboxymethyl cellulose and dextran. It was only one-third of control for agarose, zosterin, carrageenans and dextran sulfate.

Th-53

V. Ruban, V. Stepanjuk, O. Ilchenko and K. Ilchenko
Department of Viruses and Microorganisms,
Department of Industrial Microorganisms,
Institute of Microbiology and Virology of the Ukrainian Academy of Sciences, Kiev, Ukraine

The positive gram staining of representatives of the genera *Aeromonas* and *Erwinia* with a cell wall of the gram negative type and which are not referred to the gram variable one is shown to be possible. The positive staining depends on the presence of the surface regular layers of the wall (microcapsule) which is formed in cells of the ageing populations. A denaturation of the microcapsules protein in a period the gram staining, specifically under the influence of the ethanol, apparently can protect the membranes from a destruction and for this reason a stated complex do not wash from the cells. More massive accumulation of a microcapsule in the place of the cell-division or the mosaic accumulation of the peptidoglycan polymer can condition the polar coloration of the cell. Some bacteria, which have gram-positive and very thin cell-wall, hold a dark blue paint. And it more complicate the estimation of a stated gram staining. The cytological type of structure of the cell wall of bacteria may be established rather trustworthily only by electron microscopy. This is necessary to take into account in a systematization, during an identification of the bacteria and during the estimation of a purity of the culture by a method gram staining.

Imaging of Macrophages by Atomic Force Microscopy (AFM)**Th-55** Th. BAIERL, G. POLZER and A. SEIDEL,
Kernforschungszentrum Karlsruhe GmbH, Inst. f. Toxikologie,
Postfach 3640, D 76021 Karlsruhe

The surface of living cells can be imaged by the use of an AFM because it does not need conducting surfaces for imaging. We use the AFM (TMX 2010 [TopoMetrix, Darmstadt, FRG]) for analyzing the cell surface of bovine alveolar macrophages (BAM) and P388-D1 cells in aiming for molecular resolution. The cells were cultured submersed in cell medium on glass slides which were scanned either in air (but still wet) or in liquid. Compared to the pictures known from SEM, BAM in air look more flattened (up to 2.5 μm [see Fig.]). Imaged under solution the height of the cells is about 6 μm , which corresponds to the expected height of living cells (diameter of cell nucleus ~5 μm). The softness of the cell surface is the critical factor when imaging because using too high forces (>10 nN) would damage the cell membrane. Possibly for this reason we have not been able to image living P388 cells until now. Therefore cells fixed with glutaraldehyde serve as controls. These fixed cells are more rounded but no significant changes in surface properties can be recognized.

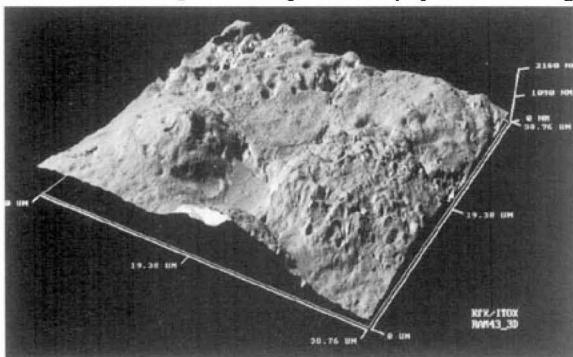


Fig.: BAM imaged in air (500 x 500 datapoints)

Th-54

RECOGNITION OF POLYMERS BY MACROPHAGES - EFFECT OF STRUCTURE
K. Smetana, Jr.^a, J. Vacík^b,
M. Jelinková^b and H.-J. Gabius^c

^aDepartment of Anatomy, 1st Faculty of Medicine, Charles University and ^bInstitute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic, ^cDepartment of Biochemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany

The polymer implant is colonized with macrophages (MPH). This study shows the effect of the chemical as well as physical structure of hydrophilic polymers on MPH behavior including endogenous lectins expression.

The results suggest the inhibitory effect of -COO⁻ groups containing monomers on the adhesion, spreading and fusion of MPH. The microporosity of polymers influenced the adhesion and fusion of MPH but not their spreading. The effect of hydrophilic polymer design on the endogenous lectins expression was only minimal.

These results show the possibility to use the synthetic polymers for study of the interaction of cells with macromolecules. They were used in the development of new advanced biomaterials for the construction of implantable devices.

Th-56 PROTEOGLYCANS FROM PORCINE FOLLICULAR FLUID AND THEIR CONTRIBUTION TO THE ACROSOME REACTION

A.Oancea ^{a)}, O.Zarnescu ^{b)},
L.Moldovan ^{a)}, D.Turcu ^{c)}.

- a) Institute of Developmental Biology, Bucharest, Romania
- b) Faculty of Biology, Bucharest, Romania
- c) National Institute of Veterinary Medicine, Bucharest, Romania

Proteoglycans were isolated from porcine ovarian follicular fluid (aspirated from small and large follicles) either by acetone precipitation or by isopycnic CsCl centrifugation in the presence of 4M guanidine HCl and protease inhibitors. Proteoglycans were then purified by gel filtration on Sepharose 2B.

Boar spermatozoa were cultured for 9h in a medium containing purified proteoglycans. Sperm were stained and examined by light microscopy for acrosome reaction degree and viability. When observed by transmission electron microscopy, acrosome-reacted sperm presented a vesiculation of the plasma and outer acrosomal membranes.

These data indicate that the proteoglycans present in follicular fluid at the time of ovulation may promote the acrosome reaction "in vitro". For this reason it is probable that the presence of follicular fluid in oviduct is very important for the ability of sperm to fertilize an ovum.

CORNEAL COLLAGEN FIBRIL FORMATION IN VITRO

Th-57

L. Moldovan, A. Dacea, M. Caloianu,
O. Gheorghiu

**Institute of Developmental Biology,
Bucharest, Romania**

The transparency of the cornea is due to the uniform diameter and regular interfibrillar distance of the collagen fibrils within the stroma. The mature corneal stroma is composed predominantly of type I collagen, but also contains a large amount of type V collagen.

We have investigated the interaction of these collagen types using an "in vitro" assay for fibril formation. Types I and V collagen were extracted from bovine corneal stroma by limited pepsin treatment and purified by salt precipitation and CM-cellulose chromatography.

Collagen fibrillogenesis was initiated by elevation of temperature and pH and studied by transmission electron microscopy. Electron micrographs showed that type I collagen formed fibrils with the characteristic 67 nm period, while type V collagen had no capacity to form striated fibrils.

Th-59 TUBULOINTERSTITIAL NEPHRITIS ANTIGEN STRUCTURE, DISTRIBUTION AND IN VITRO INTERACTIONS WITH OTHER BASEMENT MEMBRANE MACROMOLECULES

A. Charonis, R. Butkowski, E. Wayner, T. Kalfa and T. Nelson

Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota, U.S.A.

Tubulointerstitial Nephritis Antigen (TIN-ag) is a recently described 58 kD basement membrane glycoprotein, recognized by autoantibodies developed in certain patients suffering from interstitial nephritis. Amino acid sequence of peptides generated from digests of TIN-ag allowed the design of primers with which a rabbit kidney cDNA library was screened. A clone with an extended open reading frame was isolated. Sequencing data collected so far, indicate that TIN-ag has no homology to any of the known basement membrane macromolecules.

Tissue expression of TIN-ag was studied by Western Blotting and immunofluorescence, using a battery of monoclonal antibodies. These studies indicated that TIN-ag is present in low amounts in many tissues but it is found in remarkably large amounts in kidney and intestine. Isolated TIN-ag was used to study in vitro its interactions with two other major basement membrane components: laminin and type IV collagen. It was found that they both interact in a specific and saturable way with TIN-ag. Furthermore, TIN-ag was able to inhibit in a dose-dependent fashion laminin polymerization, to dissolve pre-existing laminin polymers, but had no effect on type IV collagen polymerization.

The above data suggest that the presence and the degree of expression of TIN-ag may crucially influence basement membrane structure. In this respect, it is important to note that the two sites that TIN-ag is highly expressed are basement membranes underlying epithelial layers involved in extensive transport.

SITES OF $\alpha 2\beta 1$ INTEGRIN INTERACTION ON TYPE IV COLLAGEN WHICH MEDIATE BINDING TO MESANGIAL CELLS

Th-58

E. C. Tsilibary^a, S. Setty^a, E. A. Wayner^a, and Y. Kim^b

^aDepartment of Laboratory Medicine and Pathology, ^bDepartment of Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota, U.S.A.

Type IV collagen interacts with several cell types mainly via the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin receptors. We examined the sites in type IV collagen which mediate binding to cultured human mesangial cells. These cells bound to solid phase-immobilized, intact type IV collagen (~75%), the NC1 domain (~15%), the pepsin-derived triple helical fragment (~60%), and peptide Hep-III (~25%), a discontinuity from the $\alpha 1(IV)$ [sequence: GEFYFDLRLKGDK]. In competition experiments, monoclonal antibodies against the $\alpha 2$ integrin inhibited maximally 60% of the adhesion to the intact collagen molecule and 80% of the adhesion to Hep-III, but no inhibition was observed on the NC1 and triple helical fragments. Monoclonal antibodies against the $\beta 1$ integrin inhibited almost completely (>95%) the adhesion to intact type IV collagen, the NC1 and triple-helical fragments; inhibition on Hep-III was 30%.

We conclude that $\beta 1$ integrin coupled to $\alpha 2$ and $\alpha 1$ subunits in the form of $\alpha 1\beta 1$ and $\alpha 2\beta 1$, mediates mesangial adhesion to type IV collagen, since inhibition by anti- $\alpha 2$ was at best partial. Peptide Hep-III is a major site for $\alpha 2$ integrin-mediated binding of mesangial cells to type IV collagen. The site for the $\alpha 1$ could not be assessed because no inhibiting antibodies against this subunit are available. It is possible that the $\alpha 1\beta 1$ recognizes both the NC1 and triple helical fragments, since no inhibition of adhesion was obtained on either of these proteolytic domains. Therefore, for mesangial cells, sites of integrin-mediated interactions exist on several collagenous and noncollagenous domains of type IV collagen.

RIHB EXPRESSION AND LOCALIZATION DURING EARLY CHICKEN EMBRYOGENESIS AND ITS PREDICTIVE IMPLICATION IN MORPHOGENESIS

Th-60

D. Raulais, A. Cockshutt, J.C. Jeanny and M. Vigny

Unité de Recherches Gérontologiques, INSERM U.118, 29 rue Wilhem, 75016, Paris, France.

RIHB (for Retinoic Acid Induced Heparin Binding factor) is a highly basic secreted polypeptide of the chicken extracellular matrix. It is bound via proteoglycan heparan sulfate to cell membrane as well as basement membranes. The expression of RIHB mRNA is studied by *in situ* hybridization to embryos from unincubated eggs until organogenesis. The initial ubiquitous pattern of the expression becomes more restricted to ectoderm and neural fold and notochord, then the expression remains high in regions undergoing organogenesis.

In parallel we present the protein localization during the same period of early embryogenesis, using a specific monoclonal antibody. From a patch of staining between blastodisc and vittelin, the protein staining increases with the incubation time and shows an accumulation of the protein along the future first basement membrane, even before the staining of fibronectin is detectable. The protein is then accumulated in the subsequently appearing basement membranes and around some differentiating cells during organogenesis. RIHB seems to be implicated in the establishment and/or metabolism of extracellular matrix during cell morphology, migration and differentiation.

Th-61 THE BLOOD-TESTIS BARRIER (BTB) IN PRENATALLY AND/OR PREPUBERALLY IRRADIATED RATS

J.C. Cavicchia and F.L. Sacerdote
Instituto de Histología y Embriología
5600 Mendoza, Argentina

In order to assess the influence of germ cells on the competence of the inter-Sertoli tight junctions which are the ultrastructural expression of the BTB, we prepared testes devoid of germ cells (Sertoli cell only, SCO) with prenatal ^{60}Co (one whole-body 125 rads) at intrauterine day 19. Male newborns were sacrificed, testes fixed in 5% glutaraldehyde with La(OH)₃ as an electron-opaque intercellular tracer, and processed for conventional TEM. In non-irradiated controls, zygotes are present in some tubules at 13 days. As we have shown earlier, barrier organisation first occurs in zygotene-containing tubules; at 20 days a majority of tubules display an organized BTB. In experimental SCO testes, instead, tracer freely penetrates into the tubules (BTB is absent). At 30 days the first spermatocytes appear in the irradiated, simultaneously with a competent BTB. In rats which in addition received a second dose at 20 postnatal day, we found a marked decrease in testis weight and delay in spermatocyte and BTB presence. This indicates that spermatocyte differentiation is paramount to BTB assemblage.

Th-63 EXPRESSION OF THE SARCOLECTIN BINDING PROTEIN IN THE EXTRACELLULAR MATRIX OF NORMAL SYNOVIAL TISSUE AND DURING ACUTE INFLAMMATORY EXACERBATION

H. Koepf^a, A. Zschäbitz^b, H.J. Gabius^c, and E. Stofft^b

^a St. Josefs Hospital, Wiesbaden, Germany; ^b Institute of Anatomy and Cell Biology, Mainz, Germany; ^c Institute of Physiological Chemistry, München, Germany

The interferon- α antagonist and growth promotor sarcolectin interacts with a protein (SBP) in a carbohydrate-dependent manner, that is closely related but not identical with a human macrophage migration inhibiting factor. In order to contribute to the understanding of the functional significance of sarcolectin and its tissue ligand, the distribution patterns of SBP were determined in synovial membranes from clinically affected and unaffected knee joints of patients with rheumatoid arthritis, resp. osteoarthritis, by use of biotinylated, affinity-purified sarcolectin. Binding results were compared with clinical and morphological parameters indicating inflammatory activity. In 26 of 39 specimens with histological resp. serological characteristics of inflammatory activity, SBP was immunohistologically detected in the extracellular matrix. Distribution was confined to narrow zones bordering the synovial lumen and surrounding capillaries and arterioles. Binding intensity corresponded significantly with number and activity of infiltrating macrophages. In contrast, the distribution of lymphocytes showed no relationship. Only in 2 of 18 normal synovial specimens a faint marking was detected. Expression of SBP correlated significantly with synovial fluid levels of IL-1. Sarcolectin apparently has multiple modulatory effects on inflammation as well as growth regulatory control. It could thus be integrated into complex interactions correlating inflammatory activity and proliferation of synoviocytes.

Th-62 COMPARISON OF FSH AND EGF ABILITY TO INDUCE HYALURONIC ACID SYNTHESIS BY MOUSE CUMULUS CELL-OOCYTE COMPLEXES

E. Tirone, C. D'Alessandris, G. Siracusa and A. Salustri
Department of Public Health and Cell Biology, Section of Histology and Embryology, University of Rome Tor Vergata, Italy

Preovulatory expansion of cumulus cell-oocyte complex (COC) depends on hyaluronic acid deposition in the extracellular matrix. *In vitro* synthesis of such glycosaminoglycan by cumulus cells is induced by the combined treatment with FSH and an oocyte factor(s). Among several growth factors tested *in vitro*, EGF only was able to substitute for FSH in this specific function. The aim of the work reported here was to establish if there is any interaction or correlation between them. Complexes were isolated from PMSG-primed mice and cultured for 18 h with increasing doses of FSH and of EGF. FSH gave a maximal stimulation at a concentration of 5 ng/ml, while EGF reached maximal stimulation at 0.05 ng/ml. Treatment of COCs with a combination of the two compounds at optimal doses did not promote any further increase of HA synthesis, but a synergistic effect was observed with suboptimal doses. Kinetics of FSH and EGF action was the same, both requiring about 2 h of cell exposure to induce maximal stimulation. EGF, but not FSH, action was reversed by an anti-EGF antibody. The results indicate that the stimulation of HA synthesis by FSH does not require EGF synthesis by cumulus cells as an intermediate step. On the other hand, the results are compatible with the hypothesis that EGF synthesized by other cell components of the follicle might act in a paracrine way to induce full HA synthesis in follicles exposed to suboptimal concentration of gonadotropins.

Th-64 EXPRESSION OF TENASCIN DURING THE EARLY PHASE OF HUMAN DEVELOPMENT

R. Zákány^a, L. Módus^a and R. Ádány^b

^aDepartments of Anatomy and ^bHygiene and Epidemiology, University Medical School, Debrecen, Hungary

Tenascin (TN) is considered to be an adhesion-modulating macromolecular component of the extracellular matrix (ECM). TN is absent or present only in small amount in normal adult tissues, but it can be abundantly detected during embryogenesis, when TN displays a spatially and temporally restricted tissue distribution. Transient neo- and/or overexpression of TN can be observed in healing wounds and a large variety of tumors.

Our study was designed to localize TN and follow its distribution during the first trimester of the human embryogenesis and to characterize stromal cells in the TN rich areas by immunohistochemical methods. We have found intensive TN reaction in the cartilage matrix at the very early stages of embryogenesis (4-6 weeks) which became restricted to the perichondrium at the later stages (after 9 weeks) during limb, rib and vertebra development. TN was present in a large amount in the mesenchyme of the forming skin (from 4 weeks) and also at early stages of the development of central nervous system. TN could be detected in the matrices of different organs in the intensive phase of organogenesis when it appears as an almost ubiquitous component of the ECM throughout the body. Our data obtained in double immunofluorescent systems (the detection of tenascin was combined with stainings for cell differentiation marker antigens) suggest a strong coexpression of TN with vimentin as early as the 4th week of gestation and TN could always be detected in association with proliferating epithelial cells labeled for cytokeratin. Cells positive for macrophage marker antigens (RFD 7⁺) also showed reaction for TN, and it could be detected in the intimate vicinity of cells containing desmin and smooth muscle cell actin.

Our results about morphological details of TN distribution in human embryos are essential to the better understanding not only the intricate program of human embryonic development, but the role of tenascin in newly developed matrices of tumors, as well.

Th-65

BINDING OF GLYCOPROTEINS TO PECTINS
IN PRESENCE OF CALCIUM

C. Penel and H. Greppin

Laboratoire de Physiologie végétale, Université de Genève, place de l'Université 3, 1211 Genève 4, Switzerland

Pectins are important constituents of the extracellular matrix in plants. Their backbone results from the polymerization of α -D-galacturonic acid monomers, but they contain also neutral sugar blocks. In presence of calcium, the homogalacturonan parts form 'junction zones' consisting of 2 antiparallel chains linked to the cation. We have observed that a small number of proteins exhibit a strong affinity for pectins, provided that Ca^{2+} is present. These proteins are all glycosylated as shown by their affinity for concanavalin A. Our results suggest that they adhere to pectin junction zones. This adhesion depends on the level of esterification of pectins, highly esterified polymers being inactive. Using various organs from zucchini as plant material, we have found that there are in each case 3 isoperoxidases -1 anionic and 2 cationic- among these binding proteins. These isoperoxidases have been used to study the conditions necessary for the binding to occur. It was found that it requires only a low Ca^{2+} concentration, is not modified by pH changes between 5 and 7.5, and is not affected by monovalent cations up to 200 mM. In presence of Ca^{2+} , the isoperoxidases, and other glycoproteins, bind to low concentrations of pectin (g/ml) and the resulting aggregate can be sedimented by centrifugation or separated by gel filtration chromatography. The glycoproteins can also be linked to pectins previously attached to a solid support.

The molecular interaction reported here is likely to occur *in vivo* since pectins and peroxidases are present in the same cell compartments. It could take place within the cell wall or at the surface of the cell, but also in Golgi apparatus, and be important for the targeting or the function of some extracellular proteins. Future work will show if these proteins share a common pectin binding structure.

Th-67 The Expression and Function of Heparin-binding Growth-associated Molecule (HB-GAM) in Developing Limb of the Rat

E.Szabai^a, H.Rauvala^a^aInstitute of Biotechnology, Karvaamokuja 3, P.O.Box 45, Helsinki, Finland

Heparin-binding growth-associated molecule (HB-GAM) belongs to an emerging family of developmentally regulated cytokines. It was originally purified from neonatal rat brain where it is expressed transiently but abundantly during rapid axonal growth. The expression of HB-GAM is not restricted to neuronal tissues. In this study we have shown strong HB-GAM immunoreactivity in epithelial cells and in basement membranes in the epithelial-mesenchyme interphase. In addition, HB-GAM is strongly expressed in pre-muscle cells, where it is colocalized with desmin, and in neurites innervating the limb bud, where it is colocalized with neurofilaments and with the growth associated protein-43 (GAP-43). HB-GAM seems to be associated with early events of synaptogenesis since it is closely colocalized in pre-muscle cells, with acetylcholine receptor clusters (detected with TRITC- α bungarotoxin). By using an organ culture system we showed that HB-GAM inhibits proliferation of epithelial cells. It also inhibits the mitogenic effect of bFGF on these cells. Our data suggest that HB-GAM is involved in the cell differentiation and suppression of cell division.

Th-66

cDNA CLONE OF AN AUXIN-INDUCED ALFALFA GENE CODING FOR LOW-MOLECULAR-WEIGHT PROLINE-RICH PROTEIN

J. Györgyey^a, K. Németh^a, Z. Magyar^a, T. Alliotte^b, D. Inzé^b, D. Dudit^a

^aInstitute of Plant Physiology, Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences H-6701 Szeged, P.O.Box 521, Hungary; ^bLaboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium

The plant cell wall is a complex structure of carbohydrates, proteins, lignin and other organic compounds. As an extracellular matrix it has several functions: not only mechanical support, but plays role in growth and development, intercellular communication and metabolic exchange as well.

Differential screening of a cDNA library of a synthetic auxin (2,4-D) treated alfalfa (*Medicago sativa*) callus tissues resulted in a cDNA clone called MsPRP4. Based on the sequence analysis the 630 bp long cDNA codes for a proline rich protein (84 a.a.) with a specific repeat unit of (TPVLPKR^K/RGRPPPVP). At N-terminal, a signal peptide, similar to leader sequences of extracellular proteins can be predicted. According to the Northern analysis, the corresponding gene is not, or poorly expressed in differentiated vegetative organs and somatic embryos. However there is a substantial level of expression in cultured cells and the accumulation of MsPRP4 mRNA is auxin concentration dependent. The gene belongs to the rapidly responding auxin gene family, 20 minutes after 2,4-D treatment the amount of mRNA was considerably increased. Maximum expression was observed at 24-48 hours in the presence of 2,4-D.

Considering the structural features and the expression properties of MsPRP4, this clone represents a new type of proline-rich cell wall proteins and shows characters of the typical auxin-responsive genes as well.

Th-68 ISOLATION AND PURIFICATION OF THE CYTOPLASMIC 40 kDa PRECURSOR OF THE LAMININ RECEPTOR FROM HELA CELLS

H.-P. Zimmermann, P. Ries and H. Ponstingl
Division of Molecular Biology of Mitosis, German Cancer Research Center, INF 280, D-69120 Heidelberg, FRG

We have purified the precursor of the laminin receptor from HeLa-S3 cell lysate using a specific chicken antibody. From the 70.000 x g supernatant of lysates containing 1 M NaCl, the protein was precipitated by 40% saturation with $(\text{NH}_4)_2\text{SO}_4$. The 100.000 x g pellet fraction was resuspended and fractionated by thiophilic interaction chromatography, ion exchange chromatography on SO_3^- and Mono Q, respectively. A fraction eluted from the Mono Q column at about 100 mM NaCl contains the purified antibody-positive 40 kDa protein (estimated by SDS gel electrophoresis and immunoblotting). The polypeptide was proteolytically degraded in the presence of 2 M urea with the endoproteinase Asp-N, peptides were separated by reversed phase chromatography and then sequenced. All seven residues identified corresponded to the published sequence of the human laminin receptor (H. Yow et al., PNAS 85, 6394-6398, 1988). The isolated protein binds to pure laminin in a concentration dependent manner. Immunofluorescence localization experiments using monospecific antibodies raised in chicken against the purified precursor molecule show dot-like fluorescence in the cytoplasm and at the plasma membrane of HeLa- and other vertebrate cells, whereas no fluorescence signal was detected in nuclei, correspondingly the protein was not detected on blots with proteins from isolated HeLa nuclei. The plasma membrane fluorescence could indicate a cross-reaction of the antibody with the receptor molecule, however, in Western blots exclusively a 40 kDa polypeptide was identified as the cytoplasmic precursor of the laminin receptor.

HUMAN NEUROBLASTOMA CELL ATTACHMENT
TO LAMININ IS MEDIATED THROUGH A 67-kDa
Th-69 RECEPTOR

I. Bushkin-Harav and U.Z. Littauer

Dept. of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

We have previously shown that one of the initial events in the differentiation of cultured human neuroblastoma cells is an increase in cell adhesion to the YIGSR sequence of the B1 chain of laminin (N. Garty and U.Z. Littauer, 1993, in *Neuroblastoma Res.*, No. 6). In the present study, surface receptors from adrenergic neuroblastoma LA-N1 cells were labeled by biotinylation. The solubilized particulate fraction was subjected to C(YIGSR)₃NH₂-AffiGel 10 affinity chromatography. Bound proteins were then eluted with 0.1 mg/ml of free C(YIGSR)₃NH₂, analyzed by SDS-PAGE, transferred onto a nitrocellulose membrane, and incubated with streptavidin conjugated to horseradish peroxidase. This procedure revealed one major YIGSR-binding protein of an apparent molecular weight of 67 kDa. This surface membrane protein was not eluted from the YIGSR column with an unrelated peptide, nor could it be eluted from an unrelated peptide column with the YIGSR peptide. Comparison of Bt₂cAMP-differentiated neuroblastoma cells with undifferentiated cells did not show significant differences in the level of the 67-kDa binding protein. Thus, the enhanced binding of differentiated cells to the YIGSR sequence of laminin is probably due to changes in spatial organization, or modification of the binding protein, or expression of an additional binding protein that interacts with a different site on the laminin molecule. We have recently found, however, that during the differentiation process there are considerable changes in the pattern of tyrosine-phosphorylated proteins. These findings may shed some light on the regulation of neuroblastoma differentiation and the mechanisms of laminin signal transduction.

Supported, in part, by the collaborative program between the Children's Hospital of Philadelphia and the Weizmann Institute. We would like to thank H.K. Kleinman for the gift of the synthetic peptide.

Th-71 COMPARISON OF LYtic ACTIVITIES IN VESICLES SHED BY HUMAN CARCINOMA AND FIBROSARCOMA CELLS.

A. Ginestra^a, V. Dolo^a, H. Nagase^b and M.L. Vittorelli^a.

^aDipartimento di Biologia Cellulare e dello Sviluppo via Archirafi 20, 90123 Palermo, Italy; ^bDepartment of Biochemistry and Molecular Biology, The University of Kansas Medical Center, Kansas USA.

Membrane vesicles released by tumoral cells have been suggested to play an active role in extracellular matrix degradation. As we have previously reported, human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in proMMP-9, the proenzymatic form of collagenase B, and in others lytic activities. (Dolo et al. J. Submic. Cyt. Path. in press). Now we report a zymographic comparison of gelatinolytic activities observed in vesicles shed by these cells and HT-1080, an human fibrosarcoma cell line. When cultured in high serum all cell lines shed vesicles showing a pattern of lytic activities very similar even if not identical. However, while vesicles shed in the absence of serum by breast carcinoma cell lines are almost deprived of lytic activities, vesicles shed by HT-1080 still show a lytic band apparently similar to proMMP-2.

We also report that vesicles shed by both kind of cells contain TIMP1, the tissue inhibitor of matrix metalloproteinases in high molecular weight complexes and therefore we suggest that completely inactive collagenase complexes are also present in vesicles.

This work was supported by A.I.R.C.

Th-70 A STRUCTURAL ALTERATION OF PROTEOGLYCANS OR SULFATED PROTEINS IN VARIOUS CELL CULTURES EXPOSED TO HIGH HYDROSTATIC PRESSURE

M.J. Lammi, M.O. Jortikka, T.P. Häkkinen, P. Saari, J.J. Parkkinen*, R.I. Inkkinen, H.J. Helminen and M.I. Tammi. Departments of Anatomy and Pathology*, University of Kuopio, Finland.

Hydrostatic pressure is one of the potential modulators of matrix production in articular cartilage. Previously we have shown that continuous high hydrostatic pressure (30 MPa for 20h) decreases [³⁵S]sulphate incorporation in chondrocytes (1), with simultaneous alterations in microtubular network and the Golgi complex (2), and stress fibers (3). In the presence of high pressure, altered glycosylation/sulphation of the large proteoglycans (PGs) occurred in the chondrocytes. In this study, we pressurized other cell types to investigate whether the observed alteration was specific for chondrocyte PGs.

Cells isolated from bovine fascia, synovium, and articular cartilage, and those from baby hamster kidney (BHK-21) cell line were pressurized with 30 MPa continuous hydrostatic pressure (20 h) in the presence of [³⁵S]sulphate. Media were centrifuged, ethanol-precipitated samples were electrophoresed on 1.2% SDS-agarose gels, and sulphated macromolecules were localized from the gels by autoradiography. Samples from BHK and bovine fascia cell cultures had one major sulphated band (200-300 kDa), whereas synovial cells and chondrocytes had two major bands, of which the larger was 1.2x10⁶ kDa and the smaller 200-300 kDa. In the samples from of pressurized BHK and fascia cells, the mobility of the main band was lower than in the control samples, suggesting a larger molecular size. In synovial cells both main bands had a mobility shift into higher-molecular-weight position, and in chondrocytes only the large PG, aggrecan, had a clearly altered migration.

This study shows that high hydrostatic pressure may have general effects on glycosylation/sulphation in mammalian cells. The cellular mechanism of this phenomenon needs further examination.

References: 1) Trans Orthop Res Soc 18, 47,1993; 2) Ann Rheum Dis 52,192,1993; 3) Trans Orthop Res Soc 18, 617,1993.

Th-72 STUDIES ON GAMETE INTERACTION IN Vitro BY IMMOBILIZED ZONA MATRIX

M. Mollova^a, M. Ivanova^a and J. Peknivova^b

^aInstitute of Biology and Immunology of Reproduction, Sofia, Bulgaria; ^bInstitute of Molecular Genetics, Praha, Czech Republic

Evidence has been accumulated suggesting affinity of sperm acrosomal antigens for zona pellucida (ZP). Monoclonal antibody against boar acrosin (MAb ACR.2) characterized in our earlier studies (Pernicova J., Moos J., 1990.) recognizes the 55,53, 45 and 58 kDa molecular forms of proacrosin/acrosin. In this study we used the technique for IZM described by Richardson et al. (Biol. Reprod. 45:20, 1991) to follow up the course of ZP induced acrosome reaction (AR) monitored by MAb ACR.2. The biological activity of MAb ACR.2 was also studied. The results showed visualization of 4 categories of spermatozoa interact with IZM and AR was completed in 63.79% of bound spermatozoa. Pretreatment of capacitated spermatozoa with solubilized ZP in solution led to inhibition of sperm binding to IZM (89.64%). Coincubation of capacitated spermatozoa with MAb ACR.2 and IZM resulted in inhibition of completion of AR in bound spermatozoa (36.64%) and sperm binding (93.14%) in comparison with the control system containing ascites fluid where 70.76% of bound to IZM spermatozoa manifested AR. These data suggest the role of proacrosin/acrosin in secondary binding of boar spermatozoa to ZP.

Th-73 INVOLVEMENT OF CARBOHYDRATE RECEPTOR SITES ON CAT ZONA PELLUCIDA (ZP) IN GAMETE RECOGNITION

M.D. Ivanova, M.V. Mollova, M.G. Petrov

Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Science, Sofia, Bulgaria

Carbohydrates of mammalian ZP are believed to be involved in sperm-egg recognition and interaction. Lectins, sugar binding proteins or glycoproteins of non immune origin, agglutinate cells and/or precipitate glucosconjugates having saccharides of appropriate complementarity. In our studies on cat oocyte extracellular matrix - ZP we used some lectins (ConA, DBA and WGA) and their complementary carbohydrate residues (mannose, GlcNAc, GlcNAc) for pretreatment of cat gametes coincubated for testing their binding ability. The results showed that GlcNAc and GalNAc may be a part of the so called sperm binding sites on the surface of cat ZP because the pretreatment of cat oocytes with WGA and DBA caused considerable inhibition of sperm binding. On the other hand, the preincubation of capacitated cat spermatozoa with N-acetylglucosamine and N-acetylgalactosamine led to inhibition of sperm binding to intact cat oocytes by blocking lectin specific receptors on sperm membrane.

Th-74 PHENOTYPIC CHANGES DURING SQUAMOUS DIFFERENTIATION IN TRACHEAL EPITHELIAL CELLS : THE ROLE OF EXTRACELLULAR MATRIX.

F.Marano, A.Bacza-Squiban, and E.Boisvieux, Laboratoire de Cytophysiologie et Toxicologie Cellulaire, Université Paris 7.

The epithelium which lies the airways is one of the major sites for tumor formation in human and understanding the earliest responses to injury is essential to have a clear insight into the molecular mechanisms involved in tumor development. One response is the induction of squamous metaplasia which occurs *in vivo* under environmental injuries and correspond to a repair mechanism but is also considered as a preneoplastic lesion. Primary culture of rabbit tracheal epithelial cells provides a useful tool to study the mechanisms of squamous metaplasia. The differentiation was investigated according to the nature of the extracellular matrix used. Cultures obtained by the explant technique were realized on a type I collagen substratum either as a thin dried coating or as a thick hydrated gel. Outgrowth obtained on gel was multilayered, and expressed keratin 13 (K 13), a marker of squamous metaplasia. K 13 positive cells were large upper cells with a dense keratin network. Thick gel enhanced the synthesis of the markers of hyperproliferation (K6, K16) and of the markers of basal cells (K5, K14), supporting a stratification like process which evolved toward squamous metaplasia. By contrast, the epithelium observed on coating was a monolayer of very large and spread cells with a strong expression of simple epithelium markers (K8, K18). No K13-positive cells were observed. In conclusion, these two substrata, which differ only by the structure, induce separate differentiation and appear suitable to explore the mechanisms which control respiratory epithelial cells proliferation and differentiation.

Th-75 INFLUENCE OF EXTRACELLULAR MATRIX COMPONENTS ON ACUTE PHASE RESPONSE IN RAT HEPATOCYTE CULTURES

A.Adoeva^a, I.Diakonov^a, T.Stepanova^a, A.Polevskikh^b

^aDept.of Cell Cultures, Institute of Cytology, St.Petersburg 194064; ^bDept. of Immunology, Institute of Experimental Medicine, St.Petersburg 197376, Russia.

Acid heparine-like polysaccharides consist as the main part of extracellular matrix(ECM) in liver and modulate different hepatocyte functions. Production of acute phase protein (C-reactive protein(CRP), transferrin(Tf) and ferritin(F) in rat hepatocytes(Hps) and the influence on it of the ECM components, dextran sulfate(DS) and type 1 collagen(C) were studied. Hps isolated from the liver by collagenase perfusion were cultured at 10⁵ per 0,5ml medium F12+DMEM(1:1) with 10% fetal calf serum for 2 days on uncoated or C-coated(30μg/ml) plastic surface or in the presence of DS(10μg/ml). Hps were stimulated by IL-2 (25 U/ml) or IL-1 (11 pg/ml). Production of CRP, Tf, and F was detected by cell-ELISA. It was found that DS markedly increased CRP level in the cells with the continuous fall of Tf level. Collagen slightly increased Tf synthesis while the amount of CRP decreased. The level of F decreased in the cells in all cases. Our data correspond with published materials on the acute phase response *in vivo* and point to relevance of the proposed model.

Th-76 TRANSFERRIN AND FERRITIN LEVELS IN CULTURES HEPATOCYTES ARE DIFFERENTLY CONTROLLED BY EXTRACELLULAR MATRIX

T.Stepanova^a, E.Adoeva^b, I.Diakonov^b

^aDept.of Cytodiagnostic, ^bDept.of Cell Cultures Institute of Cytology, St.Petersburg 194064, Russia

Synthesis of serum proteins involved in iron metabolism, transferrin(Tf) and ferritin(F) in rat hepatocytes(Hps) and its dependence on the extracellular matrix components (ECM) were studied. Hps isolated from the liver by collagenase perfusion were cultured at 10⁵ per 0,5ml medium F12+DMEM(1:1) with 10% fetal calf serum on uncoated or type I collagen (Coll) (30μg/ml), coated glass surface or in the presence of dextran sulphate (Ds) (10μg/ml) in the medium for 4 hours, 1, 2 and 3 days. By cytophotometry at 560nm quantitative study was performed of cell Tf and F content detected by indirect immunoperoxidase reaction using specific MAbs with 4-chlor-1 naphtol as a substrate. In the absence of added ECM components the 1,5 fold daily decrease of Tf level was found, the same was on the Coll. In the presence of Ds Tf level only slightly decreased. On the contrary, the amount of F in the cell was little changed without ECM. The addition of either Coll or Ds caused dramatic decrease of F content by the 2nd day for Coll and by the 3rd day for Ds. Found peculiarities of Tf and F levels in the cells allows to speculate about the differences in ECM-control of synthesis of these two proteins.

Th-77 EXTRACELLULAR MATRIX PROTEINS FROM TURBELLARIANS (FLATWORMS) CHARACTERIZED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

R. Peter

Institut für Genetik und Allgemeine Biologie der Universität Salzburg, A-5020 Salzburg, Austria

Extracellular matrix extracts were prepared from three species of turbellarians (free living flatworms). The pellets left after extraction of the tissues with 3.4 M NaCl were subsequently treated with 2.0 M urea (Kleinman, H. K., et al. 1986: Biochemistry 25, 312) and then with 3% sodium dodecyl sulfate containing 6% β -mercaptoethanol. SDS electrophoresis essentially followed the scheme given by Timpl, R., et al. 1979 (J. Biol. Chem. 254, 9933).

Convolutriloba retrogemma, a representative of the group of Acoela, *Dugesia polychroa* and *D. tahitiensis* (Tricladida paludicola) yielded similar patterns of collagen IV, resembling a mammalian standard, but with additional bands from 12-46 kD. Urea apparently extracted laminin from *Dugesia*, as followed from the patterns similar to mouse laminin. *Convolutriloba* lacked these fractions. In first blotting experiments, *Dugesia* showed cross reactivity with antibodies to human collagen IV and laminin. No cross reactivity with either antibody was found for *Convolutriloba*.

This is the first electrophoretic evidence of extracellular matrix proteins reported for turbellarians.

Th-79 CELL-MATRIX INTERACTIONS INFLUENCE THE PHENOTYPE OF MOUSE FETAL CHOROID PLEXUS EPITHELIAL CELLS

E. Stadler and M. Dziadek.

Institute of Reproduction and Development, Monash University, Clayton, Victoria 3168, Australia

We have previously demonstrated the importance of cell-matrix interactions in the maintenance of the polarized phenotype of 12.5 day fetal mouse choroid plexus epithelial (CPE) cells in primary culture. Cells cultured on a Matrigel substrate form polarized epithelial vesicles with an internal lumen which resemble the *in vivo* differentiated phenotype. We now show that changes in the concentration of Matrigel results in significant changes in epithelial morphology. Mixing a collagen I gel with Matrigel in a 2:1 ratio results in the formation of epithelial monolayers on the surface of the substrate, and no "invasion" of cells into the matrix. Adding TGF β 2 to the CPE cells cultured on this mixed collagen I/Matrigel substrate results in epithelial vesicle formation identical to the phenotype seen on 100% Matrigel. This suggests that the observed effect of reducing Matrigel concentration on the CPE cell phenotype is due to either a reduction in matrix-bound TGF β or reduced production of TGF β by CPE cells. Further reducing the concentration of Matrigel and collagen I in the substrate by diluting the matrix with agarose results in transformation of CPE cells at the boundaries of the epithelial monolayers into migratory mesenchymal-like cells within 4 days of culture. These data demonstrate that alterations in the composition of the extracellular matrix by either changes in the concentration of basement membrane components or the concentration of growth factors sequestered in the matrix are sufficient to cause significant changes in epithelial phenotype.

Th-78 EXPRESSION OF COLLAGEN VI mRNAs AND PROTEIN IN THE POSTIMPLANTATION MOUSE EMBRYO AND UTERUS.

P. Darling, M-L. Chu^a, R-Z. Zhang^a, T-C. Pan^a, R. Timpl^b, and M. Dziadek.

Institute of Reproduction and Development, Monash University, Clayton, Vic 3168, Australia; ^aJefferson Inst. Molecular Medicine, Thomas Jefferson Univ., Philadelphia, PA 19107, USA; ^bMax Planck Institut für Biochemie, 82152 Martinsried, Germany.

Collagen VI is a microfibrillar component of the extracellular matrix (ECM) in most tissues which has been implicated to have a major role in matrix stability and organization. To elucidate the role of collagen VI in the deciduation of the uterine epithelium after embryo implantation and in the development of the mouse embryo we have determined the pattern of expression of the three collagen VI α -chain mRNAs by *in situ* hybridization, and the localization of the corresponding protein subunits and intact collagen VI by immunohistochemistry. The uterine myometrium and endometrium contain high levels of all three mRNAs and all three α -chain subunits are localized in the ECM. Decidual cells from day 5.5 of gestation onwards do not synthesize significant levels of mRNA for any α -chain subunit except for cells adjacent to the uterine epithelium which produce $\alpha 1(VI)$ and $\alpha 2(VI)$ chain mRNA but no $\alpha 3(VI)$ chain mRNA. No collagen VI is deposited in the ECM at this site, and within the decidua collagen VI is predominantly localized within the subendothelial matrix. At day 10.5 of gestation only mesoderm cells of the visceral yolk sac synthesize collagen VI. Between day 12.5 and day 13.5 all three α -chain mRNAs are produced in mesenchymal cells throughout the embryo. The pattern of protein expression appears to parallel the pattern of mRNA expression. These data show that collagen VI is expressed at a later stage in development than collagens I and III and other components of the mesenchymal ECM.

Th-80 HUMAN SKIN RECONSTRUCTED ON SYNTHETIC POROUS MEMBRANE (INSERT). INFLUENCE OF EXPERIMENTAL CONDITIONS ON EXPRESSION OF ADHESIVE MOLECULES (INTEGRINS).

J. Font^a, M-S. Noel-Hudson^b, F. Braut-Boucher^a, J. Pichon^a, M. Robert^b, J. Wepierre^b and M. Aubery^a

^a INSERM U.180 UFR Biomédicale des Saints-Pères 75006 Paris, France; ^b Laboratoire de Pharmacologie, Unité de Dermopharmacologie, Faculté de Pharmacie, 5 rue J.B. Clément 92296 Châtenay-Malabry Cedex, France

A simplified model of cultured human keratinocytes at the air-liquid interface of a non-coated artificial membrane was developed.

In this model, besides the analysis of the well-known differentiation markers, the integrins involved in the cell-cell and cell-extracellular matrix interactions are of great interest. All basal keratinocytes express cell surface receptors of the integrin family.

In our culture system, the expression of the different integrin subunits ($\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$) was analysed as a function of the differentiation state in two different media (K-SFM or DMEM/HAM F12 supplemented with 5% FCS and adjusted at 1.5 mM calcium).

The most significant data are the preponderant expression of the $\alpha 2$ and $\alpha 3$ subunits in the basal and suprabasal layers with a membrane expression which differ according to the culture media. The terminal differentiation obtained in DMEM/HAM F12 is associated with progressive loss of the integrin expression.

The presence of fibroblasts in the underneath compartment modifies the expression of integrins: when they are proliferative, the expression of $\alpha 2$ and $\alpha 6$ chains is delayed in the DMEM/HAM F12 supplemented with FCS, and the $\alpha 6$ chain is absent in the medium supplemented with Ultronser G instead of FCS; when they are confluent or blocked by mitomycin C, greater changes are observed only in the medium supplemented with Ultronser G and lead to inhibit or delay the expression of $\alpha 2$ and $\alpha 6$. In addition, the expression of filaggrin (marker of terminal differentiation) is affected; it is delayed in the medium supplemented with FCS and inhibited with Ultronser G in the presence of proliferating and confluent fibroblasts.

RECEPTORS TO COLLAGEN TYPES I, III, IV
OF VARIOUS CELL LINES

Th-81 L.A. Sadofiev, N.A. Mikhailova

Department of Cell Cultures,
Institute of Cytology RAS, St.Petersburg,
Russia

Cell-extracellular matrix interactions are very important for most fundamental cell functions. However, there are no data about full set of receptors to extracellular matrix proteins and partially collagens in the accessible literature.

The aim of present work is to compare the full set of receptors for collagen types I, III, IV of epithelial and bone cells.

In our work we used affinity chromatography in the presence of calcium ions for purification of receptors and SDS-PAGE electrophoresis for analyses of collagen-binding proteins.

A large variety of collagen receptors was found in all tested cell lines. Among them some integrins, a receptor from 68 KD group and some low weight (43 - 20 KD) proteins were identified. We have found no remarkable difference between proteins eluted from columns with collagen I, III and IV. So, by our data, all Ca-dependent collagen receptors are able to bind each of three tested types of collagens.

We have not found principal difference in spectrum of collagen receptors between epithelial and bone cell lines. But the various groups of receptors are prevailing in various cell lines. So, the relative amount of the proteins belong to various receptor groups depends upon the cell type.

MODULATION OF CA⁺⁺-INDUCED NORMAL HUMAN KERATINOCYTE DIFFERENTIATION BY

Th-82 LIAROZOLE.HCl
G. Daneels, M. Vandermeeren, L. Bols, M. Borgers & J. Geysen.
Dept of Cell Biology / Life Sciences, Janssen Research Foundation, 2340 Beerse, Belgium.

Membrane bound transglutaminase (TGase1) is a key enzyme in keratinisation. In normal epidermis it is confined to the granular layers, but in psoriatic epidermis it is prematurely expressed in suprabasal layers (Schroeder *et al.*, 1992, *J. Invest. Dermatol.*, **99**, 27). *In vitro*, TGase1 expression can be elicited in keratinocyte cultures in high Ca⁺⁺ culture conditions and retinoic acid (RA) or synthetic retinoids suppress Ca⁺⁺-induced TGase1 expression (Michel *et al.*, 1991, *Anal. Biochem.* **192**: 232).

We combined existing microplate assays for cell viability (mitochondrial activity, MTT, Mossman, 1983, *J. Immunol. Methods* **65**, 55) with TGase1 (Michel *et al.*, 1991) to consecutively measure cell growth/toxicity and keratinisation of normal human epidermal keratinocytes in the same culture. In separate assays, RA was shown to inhibit TGase1 expression (IC50 = 8±4 nM) and to stimulate mitochondrial activity (ED50 = 40 nM) in a dose dependent fashion.

Liarozole is an inhibitor of cytochrome-P-450 mediated catabolism of RA and it exerts retinoid-mimetic effects *in vivo* (Van Wauwe *et al.*, 1992, *J. Pharmacol. Exp. Ther.* **261**: 7739). 1 µM of liarozole shifted the RA dose curves for MTT and TGase1 to 8-fold lower concentration. In the absence of RA, liarozole had no effect on MTT or TGase1, suggesting that the drug affects keratinocyte differentiation through RA. Even at 10 nM, liarozole still inhibited TGase1 expression by 20% in the presence of 0.1 nM RA. At this low dose, RA By itself does not downregulate Ca⁺⁺-induced TGase1 expression.

The data demonstrate that liarozole is a potent modulator of Ca⁺⁺-induced *in vitro* keratinocyte differentiation in the presence of low RA levels. We speculate that blocking keratinocyte RA metabolism by liarozole increases the intracellular RA levels sufficiently to initiate RA mimetic effects as exemplified by downregulation of TGase1.

INTERACTION OF KERATINOCYTES
WITH HYDROGELS

Th-83

B. Dvořáková^a, J. Vacík^b, M. Jelinková^b and K. Smetana, Jr.^c

^aPrague Burn Center, ^bInstitute of Macromolecular Chemistry, Czech Academy of Sciences, ^cDepartment of Anatomy, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

Synthetic hydrophilic polymers (hydrogels) are generally known as a very poor support for the cell cultivation. On the other hand, their physical similarity with extracellular matrix seems to be very useful for biomedical application. This paper demonstrates the first results of the development of new hydrophilic polymer support for the cultivation and subsequent grafting of keratinocytes for the treatment of skin lesion.

The keratinocytes were cocultivated with 3-T-3 irradiated mouse fibroblasts (feeding cells) on surface of wide panel of hydrogels with or without preincubation of polymers with bovine serum. The polymers containing hydroxylic and/or minimal concentration of carboxylic groups represented a very good support for the growth of keratinocytes after the preincubation of polymers in the bovine serum. The 3-T-3 feeding cells can be very well removed (in contrast to keratinocytes) by trypsinisation.

CANCELLED

Th-85 DEMONSTRATION OF VASOPRESSIN-BINDING SITES AT THE BLOOD-BRAIN BARRIER BY ELECTRON MICROSCOPE AUTORADIOGRAPHY

H. Schneider, R. Kretzschmar and A. Ermisch
University of Leipzig, Institute of Zoology, Germany

The blood-brain barrier is equipped with specific binding sites for peptides. The distribution pattern of peptide receptors at the cellular level remains to be elucidated, e.g. by electron microscope autoradiography. We found a saturable binding of arginine-vasopressin (AVP) at the blood-brain barrier both, *in vivo* using the intracarotid bolus injection technique and *in vitro* by radioligand binding studies on isolated brain microvessels. Here we used isolated capillaries of rat hippocampus which were incubated with ^{125}I -AVP and then fixed with glutaraldehyde. In electron microscope autoradiographs of single capillary cross-sections silver grains were present at the luminal and abluminal side of endothelial cells and were also associated with pericytes. In a competition experiment the specific binding of ^{125}I -AVP to different cellular compartments has been analyzed by silver grain counting (50 % "probability circle" analysis). The method allows to investigate single cross-sections of capillaries without any surrounding material. This avoids background noise in activity measurements. The low concentrations of AVP applied could be detected on the electron microscopic level by using radioiodinated AVP. As a result of isolation and fixation procedure cells are swollen. This permits to assign single silver grains to different compartments with respect to the limited resolution of the technique (resolution circle diameter = 270 nm).

There is an indication for specific AVP-binding

- a) at the luminal side and
- b) at the abluminal side of endothelial cells as well as
- c) at pericytes.

Two further competition experiments are currently analysed.

MITOCHONDRIAL tRNA PROCESSING

Th-87 IN MAMMALS

W. Rossmanith^a, A. Tulle^b, T. Potuschak^c, R. Karwan^a
and E. Sbisà^b

^a Institut für Tumoriologie-Krebsforschung der Universität Wien,
Borschkegasse 8a, 1090 Wien, ^b Centro di Studio sui Mitochondri e
Metabolismo Energetico, CNR Bari, Via Orabona 4, 70125 Bari,

^c Institut für Botanik der Universität Wien, Rennweg 14, 1030 Wien
In vertebrate mitochondria each strand of the circular genome is transcribed from major promoters into polycistronic RNAs. Since most of the mRNA and rRNA coding sequences are immediately contiguous to a tRNA gene, the processing of primary transcripts is likely to be carried out by pre-tRNA processing enzymes. Although this "tRNA punctuation model of RNA processing" gains support from the identification of processing intermediates, no vertebrate mitochondrial activity that cleaves an authentic mitochondrial tRNA precursor has been described.

We report the precise endonucleolytic processing of mammalian mitochondrial tRNA precursors with HeLa cell mitochondrial extracts. We show that the mitochondrial tRNA processing activity/ies and nuclear RNase P have distinct molecular and enzymatic properties. Data on the characterization of the processing products, their further maturation, and the activity/ies involved will be presented.

This work is supported by the Austrian Science Foundation, by the HSJS der Stadt Wien, by the BMWF (ÖAD #16), by Progetto "Finalizzata Ingegneria Genetica" CNR, Italy and by MURST, Italy.

MOLECULAR ANALYSIS OF CHLOROPLAST DIVISION

Th-86 R. Reski, B. Kasten, S. Kruse, R. Reuter, M. Faust, S. Rother, S. Quast, W. O. Abel
Institut f. Allgemeine Botanik, D-22609
Hamburg, Germany

The molecular events underlying chloroplast division are studied with the mutant PC22 of the moss *Physcomitrella patens*. This particular mutant is defective in chloroplast division thus possessing at the most one giant chloroplast per cell. This macrochloroplast exhibits normal internal fine structure, but is severed by the enlarging cell plate during cytokinesis. Its division can be induced by cytokinin within hours. Likewise, the phytohormone promotes maturation of complex plastid transcripts and a transient occurrence of plastid polypeptides. These cytokinin-induced molecular changes interfere with other internal and external stimuli, e.g. blue light and endogenous oscillators. Detailed analyses utilizing two-dimensional electrophoresis (IEF/SDS-PAGE) reveal a cascade of plastid proteins underlying chloroplast division in this particular mutant. Identification of these proteins by Western-analyses and microsequencing will be reported. Restriction enzyme- and Southern-analyses gave no hints for mutations in the plastid DNA of this chloroplast mutant but revealed its methylation around an open reading frame (ORF), possibly encoding a zinc-finger protein. This ORF is conserved from prokaryotes to the plastids of archegoniates but is absent from the plastids of monocotyledonous plants. In *Physcomitrella* it is preceded by a complex promoter structure, so far unknown for plastid genes. Expression of the protein and analysis of its function will be reported. Four cDNAs representing novel genes have been isolated by molecular subtraction. Their expression is developmentally regulated and respond to cytokinin within one hour. Expression of these genes and behaviour of the mutant macrochloroplast was analysed in somatic hybrids and in transgenic plants overproducing cytokinin.

Acknowledgements: This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Commission of the European Community.

TRANSMEMBRANE ADHESION PROTEINS AND CATENINS: A CRITICAL ROLE IN UTERINE EPITHELIAL CELL BEHAVIOUR

M. Thie, P. Fuchs, A. Albers, and H.-W. Denker
Institute of Anatomy, University of Essen, Medical School, D-45122 Essen, Germany

Human uterine epithelium displays a distinct polarized organization as typical for all simple epithelia with apical, lateral, and basal membrane domains. Although non-adhesive throughout most of the menstrual cycle, epithelial cells (ECs) allow attachment of trophoblast cells to their apical plasma membrane during embryo implantation. A recent hypothesis postulates that ECs turn off genes for apical-basal polarity and turn on genes for a more fibroblast-like phenotype which is adhesive for trophoblast. Insight into the regulation of polarization and epithelial-mesenchymal transformation in this system should be gained from studies using non-adhesive and adhesive uterine EC lines. (1) HEC-1A which are non-adhesive for trophoblast appear highly polarized with respect to the spatial organization of the cytoplasm. The lateral membranes form tight junctions, zonulae adherentes, and desmosomes. Cells express transmembrane adhesion proteins (TAPs: α_6 , β_1 , β_4 integrin subunits and E-cadherin), both present at the lateral membrane of adjacent cells. Intracellular proteins (α , β -catenins) are colocalized with these proteins indicating functional interaction. (2) RL95-2 which are adhesive for trophoblast appear non-polarized. Although TAPs are expressed conventional intercellular junctions are not formed. TAPs and β -catenin are localized on all membrane domains. α -catenin, however, is not expressed. (3) AN3-CA are non-adhesive for trophoblast and appear non-polarized lacking formation of organized intercellular junctions. These cells are deficient in the expression of TAPs (β_4 integrin subunit; E-cadherin) and β -catenin. Our data suggest that the expression of a well-polarized phenotype of uterine ECs depends critically on formation of a complete E-cadherin - catenin complex and that lack of expression of a single component can suffice for blocking polarization. We hope to get insight into the master gene program for the epithelial phenotype using these uterine EC lines.

Th-89 EVIDENCE FOR Ca^{2+} -DEPENDENT CONFORMATIONAL CHANGES OF CALRETININ BY LIMITED TRYPTIC PROTEOLYSIS

J. Kuźnicki^a, L. Winsky, B. M. Martin, T.-C.L. Wang, D. M. Jacobowitz

Laboratory of Clinical Science, NIMH, Bethesda, Maryland

The tryptic digestion pattern of rat recombinant calretinin was dependent on Ca^{2+} as determined by analysis of the fragments by SDS-gel electrophoresis, amino acid sequence and electrospray mass spectroscopy. Ca^{2+} -saturated calretinin was cleaved between amino acids 60-61 to yield two fragments, which accumulated during cleavage. Small amounts of 61-271 were further cleaved yielding fragments 61-170 (unconfirmed), 61-189, and 61-233. Calretinin was also cleaved between residues 60-61 in the presence of EGTA. However, under those conditions, a cleavage of fragment 61-271 occurred more readily and from the N-terminal end, yielding peptides 68-271, 70-271, 75-271 and 178-271. All identified fragments were able to bind $^{45}\text{Ca}^{2+}$ on nitrocellulose filters, but to different extents. The results indicate that there are sites which are available to trypsin regardless of Ca^{2+} -binding (60-61), sites which are completely protected against trypsin upon Ca^{2+} -binding, e.g. first α -helix of the II EF-hand domain, and sites which are partly available upon Ca^{2+} -binding such as 189-190 and 233-234. All these data show that calretinin changes its conformation upon Ca^{2+} -binding.

* permanent address: Nencki Institute, Warsaw, Poland

Th-91 FORMATION OF SPHEROIDS, WATER TRANSPORT, AND ULTRASTRUCTURE OF LLC-PK1 CELLS IN CULTURE
E.I. Christensen^a, S. Nielsen^a, H. Vik^b, and K.J. Andersen^c.

^aDepartment of Cell Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark; ^bInstitute of Diagnostic Radiology and ^cMedical Department A, University of Bergen, Haukeland Hospital, Bergen, Norway.

LLC-PK1 cells of renal proximal tubular origin started to form spheroids within 24 hr in a serum containing medium and within 2-3 days in a chemically defined medium, when grown in culture flasks previously base-coated with agar (0.75%). The multicellular spheroids, with an average diameter of 100-350 μm , were free floating and showed a butterfly-like structure due to the formation of several hollow microspheres. These were lined with polarized epithelial cells which had an abundance of microvilli protruding from the apical surface into the external medium, and a well developed vacuolar apparatus including coated pits, endosomes and lysosomes. The microspheres were sealed between lumen and the surrounding medium by tight junctions and were found to fluctuate in size due to fluid being transported in an apical to basal direction. The addition of ouabain and mercury chloride inhibited these fluctuations.

In conclusion, the LLC-PK1 spheroids represent a biological system of intermediate complexity between renal tissue *in vivo* and simple monolayer cultures, providing a tool for studying renal physiological mechanisms in general, including water and solute transport, and also provide an excellent model for studying the potential nephrotoxicity of drugs and chemicals.

Th-90 TRANSCYTOSIS OF SOLUBLE MOLECULES FROM THE BASOLATERAL TO THE APICAL POLE OF THYROID EPITHELIAL CELLS ORGANIZED IN FOLLICLES

V.Giré, Z.Kostrouch, F.Bernier-Valentin, and B.Rousset.
INSERM U.369, Faculté de Médecine A. Carrel 69372 Lyon-France.

Endocytosis is known to occur from both the apical and the basolateral surfaces of polarized thyrocytes. Part of solute molecules internalized by apical endocytosis are transcytosed to the opposite cell surface. In the present study, we have analyzed the basolateral to apical transcytotic activity of polarized thyrocytes. We have measured the transport of both fluid phase markers (Lucifer Yellow or LY and Dextran) and proteins (thyroglobulin or Tg and BSA) from the extracellular medium to the follicular lumen of *in vitro* reconstituted thyroid follicles (RTF). Three types of tracers were used: a) BSA and Tg coupled to colloidal gold particles. b) Dextran (10 to 70 KDa), BSA and Tg labeled with FITC: D-F, BSA-F, Tg-F respectively and c) radioiodinated Tg and BSA.

Tg-gold and BSA-gold complexes were first detected in coated vesicles and early endosomes (within 5 min) and then in electron dense vacuoles (prelysosomes and lysosomes). After 120 min, few particles were detected in the lumen of RTF. Videomicroscope analyses revealed that BSA-F and Tg-F mostly accumulated in the cell monolayer constituting the follicles whereas D-F or LY were quasi-exclusively detected into the lumen of RTF. The D-F content of lumena linearly increased with time (up to 5h), was proportional to the concentration of the probe introduced into the medium, was temperature-dependent (blocked at 17°C) and activated by thyrotropin in a concentration dependant manner. Using radioiodinated Tg and BSA, we found that only a limited fraction of basolaterally endocytosed Tg and BSA were transported in lumena. After 8h, 8% of Tg and 30% of BSA were recovered in lumena.

Our data show that solute molecules that enter thyrocytes by basolateral endocytosis are subjected to different intracellular routing. Fluid phase markers are efficiently transcytosed whereas proteins are mainly conveyed to late endosomes and lysosomes.

Th-92 DISTINCT APICAL (B10) AND BASOLATERAL PROTEINS WITH ALKALINE PHOSPHODIESTERASE I ACTIVITY ARE PRESENT IN RAT HEPATOCYTES

L. Scott, D. Delautier, G. Trugnan and M. Maurice
INSERM U 327 and U 410, Faculté de Médecine Xavier Bichat, Paris, FRANCE

B10 is an apical plasma membrane (PM) protein found in rat hepatocytes (130 kDa) and intestinal epithelial cells (120 kDa). To determine the identity of B10, 3 CNBr cleaved peptides from the liver purified protein were sequenced. One had a 70% homology to human PC-1, a transmembrane protein which has alkaline phosphodiesterase I (APDE) activity and a basolateral distribution in many epithelial cells. Anti-B10 antibodies immunoprecipitated approximately 90% of the APDE activity from intestinal homogenate, but only 40% from hepatocyte PMs. To investigate the PM localization of the unprecipitated APDE activity in hepatocytes, we separated total PM vesicles on a sucrose density gradient. The APDE activity precipitated by anti-B10 had an apical distribution, whereas the majority of the remaining activity was basolateral.

Intestinal B10 was partially cleaved by phosphatidylinositol phospholipase C, whereas the liver molecule was not cleaved. However, because these two molecules had the same molecular weight following deglycosylation with Endo F, it is possible that the liver molecule contains an uncleavable glycosyl-phosphatidylinositol anchor. In conclusion, at least two proteins with APDE activity but with different plasma membrane localizations, and therefore different targeting signals, are present in the hepatocyte. We are currently investigating the relationship of the liver and intestinal B10 to each other and to PC-1 by molecular cloning.

Th-93 THE TRANSMEMBRANE DOMAIN OF AMINO-PEPTIDASE N IS NECESSARY FOR EFFICIENT TRANSCYTOSIS IN CACO-2 CELLS.
L. K. Vogel, O. Norén and H. Sjöström.

Department of Medical Biochemistry and Genetics,
Biochemistry Laboratory C, University of Copenhagen, The
Panum Institute, Blegdamsvej 3, 2200 Copenhagen N,
Denmark FAX 45/35367980.

In Caco-2 cells aminopeptidase N is localized at the apical membrane and known to be transported by the indirect pathway e.g. from the trans Golgi network via the basolateral membrane to the apical membrane. This study aims at identifying sorting signals that are necessary for this pathway. Two secretory forms of the human aminopeptidase N, known to be sorted apically in MDCK cells, were expressed in Caco-2 cells. Both forms were secreted approximately equally to both sides of Caco-2 cells. This shows that the transmembrane and/or the cytoplasmic sequences are necessary for efficient sorting to the apical membrane in Caco-2 cells. cDNA constructs coding for porcine aminopeptidase N mutated in the only cytoplasmic tyrosine (tyr6) or containing a deletion of the cytoplasmic part (tailless) were constructed and expressed in Caco-2 cells. Both tyr6 and tail-less forms are located at the apical surface in a manner indistinguishable from wild type porcine aminopeptidase N. In addition we have shown that the tail-less aminopeptidase N is transcytosed from the basolateral side to the apical side as is wild type aminopeptidase N. We therefore conclude that membrane anchoring possibly by an intramembrane signal is necessary for efficient sorting to the apical side of Caco-2 cells.

Th-94 LACK OF CORRELATION BETWEEN IgG INTERNALIZATION AND PLACENTAL ALKALINE PHOSPHATASE EXPRESSION

I. Stefaner ^a, L. C. Kamel ^b, W. Hunziker ^b and R. Fuchs ^a

^a Dept. Gen. Exp. Pathology, Univ. of Vienna, Austria; ^b Inst. Biochemistry, Univ. of Lausanne, Switzerland.

Transfer of IgG from the maternal circulation to the fetus is an important mechanism by which primates provide their offspring with passive immunity. This process is thought to involve syncytiotrophoblast-specific IgG Fc receptors (FcR) that bind maternal IgG at the apical surface and selectively transcytose it to the basolateral plasma membrane with subsequent release into the fetal circulation. Recent studies by Makyia and Stigbrand (Biochem. Biophys. Res. Comm. 182, 1992, 624-630) have proposed that placental alkaline phosphatase (PLAP), a glycosyl-phosphoinositol (GPI) anchored protein, serves this function.

To analyze the possible role of PLAP as an IgG receptor, we used several cell lines expressing PLAP endogenously or following transfection of the cDNA. MDCK cells expressing PLAP did not show increased IgG binding or internalization as compared to non transfected cells at IgG concentrations ranging between 50 mg to 5 mg/ml. Similarly, BeWo and Hep-2 cells, which both endogenously express PLAP, did not internalize significant amounts of IgG. Furthermore, upregulation of PLAP expression in BeWo cells using methotrexate did not affect this amount of cell-associated IgG. In contrast, however, anti-PLAP antibodies were bound at 4°C and internalized upon warming to 37°C by PLAP-expressing cells. Taken together, these results do not support a role of PLAP in IgG binding and transcytosis.

This work was supported by the Austrian Science Research Fund P9499 (R.F.) and the Swiss National Science Foundation (W.H.).

Th-95 MATING BEHAVIOUR OF YEAST CELLS AND PROTOPLASTS

I. Pokorná and A. Svoboda

Department of Biology, Faculty of Medicine, Masaryk University, 662 43 Brno, Czech Republic

The mating reaction between cells and protoplasts of *Saccharomyces cerevisiae* was studied by phase contrast and fluorescence microscopy. Mating morphology in each form was different. The protoplast in the vicinity of a cell of the opposite mating type first grew without spacial orientation, later its growth ceased and the protoplast became spherical. The cell sent a mating projection towards its partner protoplast but, even if this touched the protoplast surface, fusion did not result. The absence of mating morphogenesis in the protoplast contrasted with the behaviour of its nucleus and cytoskeleton. The single nucleus migrated towards the plasma membrane to the site of protoplast-cell contact, to which also the cytoplasmic microtubules were directed. Actin patches also accumulated in that region. Identical changes could be observed in the cytoplasm of protoplasts incubated with alpha-factor. These findings suggest that protoplasting did not interfere with the receptor for pheromone. Contact between the cell wall and protoplast plasma membrane initiated neither breakdown of the wall nor fusion of the cytoplasm. This implies that, at mating, the cell wall plays a key role in controlling polarized growth as well as in fusion of the partners.

Th-96 A ROLE FOR ACTIN PATCHES IN THE SYNTHESIS OF WALL COMPONENTS IN YEASTS

M. Gabriel and M. Kopecká

Department of Biology, Faculty of Medicine, Masaryk University, 662 43 Brno, Czech Republic

In budding and fission yeasts, actin patches are present in areas of intensive wall synthesis. Using two different model systems - yeast protoplasts and ts actin mutants in which delocalization of actin patches is induced by protoplasting and actin gene mutation, respectively - we present evidence of general validity of structural and probably even functional relations between actin patches and the wall synthesis.

In regenerating protoplasts in the period between the onset of wall synthesis and the completion of the wall, actin patches were evenly distributed under the whole protoplast surface. After the wall completion, actin patches disappeared. With a start of polarized growth, actin patches accumulated in growth regions. In the cells of ts actin mutants growing under permissive conditions, actin patches showed asymmetric arrangement. At restrictive temperature, cells grew isodiametrically. Actin patches were evenly distributed under the whole surface. These isodiametrically growing cells produced a new wall all over the surface.

Regions involving the synthesis of wall components always showed the presence of actin patches. When the wall ceased growing, actin patches disappeared. This implies that actin patches must be closely related to the process of wall component synthesis. Actin patches may act as activators of glucan and chitin synthetases associated with the plasma membrane.

**ULTRASTRUCTURAL CHARACTERISTIC OF
Th-97 RENOMEDULLARY INTERSTITIAL CELLS
IN ENDOTOXEMIA**

N.G.Kharlanova, Yu.M.Lomov, E.A.Bardakhchian
Laboratory of Pathomorphology, Plague Research
Institute, Rostov-on-the Don, Russia

In initial period of endotoxin shock (ES) in rats and dogs takes place significant decreasing of lipid granules (LG) - prostaglandins' (PGs) precursors in the cytoplasm of renomedullary interstitial cells (IC). LG displace to IC plasma membrane or its branches, contacting with capillaries and tubules basal membranes. In intermediate period of ES hyperfunction of IC changes by dystrophic disturbances and total reducing of LG in the cytoplasm of IC and its processes. On the stage of later endotoxemia are restore a number of LG and takes place reactive changes of intracellular organelles.

It is suggested that PGs shifts in ES may be connect with synthesis, accumulating and releasing its precursors out of IC.

Th-98 ELECTRON-MICROSCOPIC FINDINGS IN *Helicobacter pylori* INFECTION OF ANTRAL MUCOSA
S.Yu. Lomov

S.Yu.Lomov
Railway Hospital, Rostov-on-the Don, Russia

Electron microscopic investigation of the gastric mucosa have been revealed spiral forms of bacteria identified as *Helicobacter pylori* (HP). Besides that there were coccoid forms as well. The mucus gel layer in patients with HP gastritis is getting thiner and microorganisms were localized under "mucus blanket" or on the epithelial cells surface. In some cases especially in the area contact HP with epithelial cells its apical plasma membrane slightly raised and formed the so-called "adhesion pedestal".

In the mucous cells there are signs of vacuolar dystrophy, oedema and HP presence. In the parietal cells bacteria penetrate through the secretory channels. In the chief cells, producing the pepsinogen, take place high secretory activity. Changes in the epithelial cells combined with infiltration of stroma by polymorpho-nuclear leucocytes, lymphocytes and plasmacytes.

Th-99 ELECTRICAL CHARACTERISTICS OF MOLLUSC BRAIN NEURONES EXPOSED TO CRYOPROTECTANTS AND DEEP FREEZING

N.Chekurova, E.Gahkova
Institute of Cell Biophysics RAS, Pushchino,
Moscow Region, Russia

The effects of DMSO, ethylene glycol and formamid (0.1M- 0.5M) on electrical parameters of snail neurone membrane were studied. It has been shown, that these substances in concentration 1-1.5M at room temperature cause membrane potential depolarisation (in average by 5-20 mV), decrease ionic membrane currents (by 20-50%) and increase or decrease input resistance of cells. All electrical parameters of neurone membrane recovered after wash out.

In another of experiments these substances were used as cryoprotectants for freezing of brain preparations from fresh-water mollusc *Lymnaea stagnalis* and sea snail *Aeolis pappilosa*. The conditions of cryoprotectants application were selected by means of electrophysiological methods: concentration of cryoprotectants was 1.5M, the time of cells exposition in the solution with cryoprotectant was 15-20 min at temperature 20°C. The preparations of isolated brains were frozen in liquid nitrogen vapor at a rate of 500°C/min. Then the samples were plunged for storage into liquid nitrogen. Thawing was performed in a water bath at 23-25°C.

Thawed neurones retained high viability and manifested normal electrical parameters even after storage in liquid nitrogen for five years.

Th-100 THE INVESTIGATION OF ENVIRONMENTAL STRESSES AND PLANT CELLS TOLERANCE
L.Sergeeva,A.Martynenko,V.Trukhanov
Institute of Plant Physiology and
Genetics of Academy of Sciences of
Ukraine. 252022
Vasilkovskaja 31/17, Kiev,Ukraine.

Our manipulations with cultured tobacco cells have resulted in the isolation of a number diverse cell lines. Variants, resistant to salt and water stresses and to some heavy metals (W,V,Zn,Cu,Ba) have been obtained.

Salt-resistant cell lines were grown about five years on MS medium plus 35 g/l sea water salts or 40 g/l sodium sulfate. Salt-adapted lines are characterized by universal resistance both to the former and to the latter type of stresses. Tolerance of long term tobacco tissue cultures is maintained by vacuolar compartmentation of salt with the subsequent osmotic compensation in the cytoplasm by proline.

Callus cultivars resistant to modelling water stress are cultured more than four years on solid medium with the addition of 250 g/l mannitol. Mannitol-stressed cultures are characterized by lowered of osmotic potential and increased level of proline. Salt- or mannitol-resistant cell lines tolerate alternative isosmotic stress.

Lines resistant to various heavy metals have been selected. Tungsten-adapted cell clones are cultured on solid nitrate-free media plus 1.5 or 2.0 mM of W. Zinc-resistant and copper-resistant variants challenge 2.0 and 0.2 mM of toxic agents accordingly. All selected lines maintain the stable growth under stress conditions. Cu-affected resistant tissue cultures are characterized by the accelerated growth. It is possible connects with the alteration of membrane permeability.

Fertile plants were regenerated from salt- and mannitol-resistant lines. They retain stable osmotic tolerance. Their seedling progeny are investigated.

Th-101

ACTION OF ESTRADIOL ON THE OVARIECTOMIZED RAT UTERUS IN CHRONIC STRESSFUL CONDITIONS
 A. Gunin, A. Sharov, A. Ivanov, A. Joshi
 Department of Histology & Biology of Medical Faculty of Chuvash State University, Cheboksary, Russia

Influence of chronic stress on realization one of the main estrogen effects in the uterus - induction of proliferation was studied. Mitotic activity of uterine structures in 24, 36, 48 hours after single injection of estradiol dipropionas (10 micrograms/rat, i.m.) in chronically stressed (swimming and overpopulation) and in control unstressed ovariectomized rats was determined by counting Mitotic Index (number of mitoses on 1000 cells). Proliferative intensivity of luminal and glandular epithelium of endometrium of chronically stressed rats in 24 and 36 hours after estradiol administration were high than data of unstressed estradiol treated rats. To 48 hours the values of Mitotic Indexes in these structures became some low than in control. Mitotic activity of endometrial stromal cells of chronically stressed and estradiol treated rats in 24 hours time was some large, in 36 hours time some low, and in 48 hours time was hiher than the data of unstressed estradiol treated rats.

Th-103

THE ROLE OF FREE RADICAL PEROXIDATION IN CELL STRESS
V.A. Baraboy^a, S.I. Zhadko^b

^aInstitute of Oncology and Radiology, 252022 Kiev, Ukraine; ^bInstitute of Botany, 252004 Kiev, Ukraine

Free radical lipid peroxidation (LP) in the membranes of the plant and animal cells is developed under the influence of various stress agents (SA). The biological role of early stress activation of LP is in mobilization of the cell adaptive reactions. The important chain in the adaptive reactions is an enhance of antioxidation activity (AO) in cells that leads to an increase of their common unspecific resistance. Under influence of strong and long-term SA a permanent decrease of AO occurs. As a consequence of this process, there is an induction of both the secondary LP activation and development of oxidative membrane destruction, and cells die in a result.

Thus, there phases are distinguished in the mechanism of cell stress: I - the primary stress activation of LP and AO mobilization; II - the relative stabilization of LP=AO equilibrium; III - the secondary LP activation. LP activation has predominantly a signal function (activation of stress-realizing systems) in the first stage, and it plays a destructive role in the third stage.

Th-102

CHLOROPLAST ULTRASTRUCTURE IN CHLORELLA CELLS IN MICROGRAVITY
 A.F. Popova

Institute of Botany, Academy of Sciences, 252004 Kiev, Ukraine

The ultrastructural organization of Chlorella vulgaris (strain LARG-1) cells grown in microgravity in darkness during different period has been studied. This strain is capable to syntheze a chlorophyll without light. The rearrangements in submicroscopic organization of energetic organelles, in particular of the chloroplasts, were found as compared to the control. A considerable reduction of reserve polysaccharides in their stroma and around a pyrenoid, the decrease of stroma electron density and relative thylakoid volume, the curves of the thylakoid bundles, the enlargement of intrathylakoid space, the appearance of vesicles and electron transparent zones in chloroplasts were found. A positive correlation has been established between the amount of reserve polysaccharides in chloroplasts and ratio of the number of soluble and structurally bound forms of amylases and the level of enzymic specific activity. The spectrum of chloroplasts rearrangements depends on the nutrient medium content and duration of Chlorella cell cultivation in microgravity. The ultrastructural data of chloroplasts are discussed in connection with changes in cell carbohydrate and protein exchanges in microgravity.

Th-104

AN ULTRASTRUCTURAL STUDY OF EFFECTS OF CYCLOHEXIMIDE, ACTINOMYCIN D AND MICROGRAVITY ON PROTONEMA CELLS

E. M. Nedukha

Department of Cell Biology and Anatomy, Institute of Botany, Kiev, Ukraine

We established likeness of biological effect of long microgravity and clinorotation to structure changes of plant cells and the presence of lability series of ultrastructure organelles change: vacuolization of cells > plastids > peroxisomes > cell wall > mitochondria > endoplasmic reticulum > apparatus Golgi > nucleus > plasmalemma > tonoplast. For study of the mechanism of microgravity influence were investigated effects of cycloheximide (0,3-1,0 mg/ml) and actinomycin D (1,0-3,0 mg/ml) to Funaria hygrometrica moss protonema cells and effects of clinorotation to cells grew on horizontal clinostat (2 rev/min) during 30 days by electron microscopy method. It was revealed that inhibitors during 5 days provoke changes of cell ultrastructure which are analogous of structure changes in cells during 30 days in clinostation. These influences were expressed in reduction of ribosomes and polyosomes density, breaking of plasmalemma and endomembranes, swelling of chloroplast thylakoids and reduction starch in plastids, thinning of cell wall. The data suggest that both transcription and translation change take place under long microgravity influence.

**EFFECT OF TIBA ON ULTRASTRUCTURE AND
IONIZED CALCIUM LOCALIZATION IN PEA
Th-105 ROOT STATOCYTES**

N.A.Belyavskaya

Institute of Botany, Kiev, Ukraine

The role of an auxin transport system in the regulation of a plant gravitropic function was studied on pea roots which were exposed in 2,3,5-triiodobenzoic acid (TIBA) solutions, an inhibitor of IAA transport, with different concentrations. Exogenous TIBA has retarded or inhibited completely growth characteristics and a gravitropic function of pea roots. It was also shown at the cellular level that this inhibitor has resulted in the disturbance of a polar topography of statocytes' organelles, the significant increase of a vacuolar compartment, the decrease of a starch content in amyloplasts and others. The intensity of a cytochemical reaction to ionized calcium was moderate. The precipitate of a calcium pyroantimonate was observed in many organelles, but it was practically absent in statocyte hyaloplasm. The data presented suppose that the loss of a gravitropic ability by roots under their treatment by TIBA may be a result of the desolation of a root mobile IAA pool; it may blockade the calcium mobilization from its intracellular stores during a seedling gravistimulation.

**Th-107 INTRACELLULAR CONTROL OF THE ISOLATED
CELL REGENERATION**

O.Demkiv, Ya.Khorkavtsiv, O.Karash

Department of Plant Ecomorphogenesis,
Institute of Ecology of Carpathians of the Academy of Sciences of Ukraine, 290000 Lviv,
Stefaniv Street, 11

Processes of recovery of the metabolic activity have been investigated in one-, two-, or three-cell complexes after their being isolated from protonema leaves of the moss *Tetraphis pellucida* Hedw. In response of the isolation a set of new reactions such as rapid change of membrane permeability, increase of calcium amount, alkalinization of the cytoplasm and other ones are switched on. The pH_{cyt} level and calcium content did not show further increase during a subsequent 2- to 3-h period. Later, the pH_{cyt} and the intensity of calcium fluorescence declined gradually and reached the initial level by the 20th hour of cell isolation. We have observed a secondary peak in the pH_{cyt} and calcium content, activity of the glucose-6-phosphate dehydrogenase between the fifth and the seventh days, that is at the stage of cell apical growth and mitotic activity of isolated cells. As well as the moss protonema development the regeneration of isolated cells has been shown to be hormonal dependent. IAA favours the alkalinization of the cytoplasm and an increase of the calcium. The action of ABA is also connected with calcium intensification, pH_{cyt} however being decreased. These changes observed appear to be of universal validity, thus providing some insight into fundamental metabolic and morphogenetic processes.

**Th-106 PROTEOLYTIC MODIFICATION OF RAT
LYMPHOCYTE ACTIN IN RESPONSE TO
RESTRAINT STRESS**

T. Sklyarova and O. Denisenko

Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russia

In the lymphocytes of rats subjected to intermittent restraint stress a new major protein migrating close to actin on two-dimensional gel-electrophoretic map was detected. Western blot analysis of total lymphocyte proteins using anti-actin polyclonal antibodies has revealed this protein to be actin. However, it had a lower molecular weight (~41kDa) and more acidic isoelectric point (pI~4.8). Peptide mapping of the two actin forms showed that the peptide patterns were identical, but each sample contained one unique peptide that was not observed in the other. We suggest that the abnormal actin form is the result of proteolytic cleavage of C-terminal 10 amino acid peptide, since this peptide contains three positively-charged amino acid residues and its cleavage from the initial actin molecule would result in the above mentioned shift in pI and Mr values. Proteolytic activity selectively modifying actin was found to be EDTA-sensitive. The changes in actin structure correlated with the increased level of endogenous ADP-ribosylation of proteins with Mr~21-24kDa in lymphocyte lysate. The modified actin form was also present, albeit as a relatively minor component, in the lymphocytes of control rats. Therefore, we conclude that such proteolytic cleavage of actin normally occurs in the organism but can be intensified by physiological stress. We suggest that the cells with truncated actin could be condemned to programmed cell death.

**Th-108 THE IMPACT OF DESTRUXIN E ON
SUBCELLULAR DISTRIBUTION OF ELEMENTS
IN CELLS OF MALPIGHIAN TUBULES OF
WAX MOTH LARVAE, *GALLERIA MELLOMELLA***

Z. Žižka^a, R. Pelc^a, C. Dumas^b and A. Vey^b

^aInstitute of Microbiology, Academy of Sciences, Vídeňská 1083, 14220 Praha 4, Czech Republic; ^bStation de Recherches de Pathologie Comparée I.N.R.A., URA C.N.R.S. 1184, 30380 Saint Christol Lez Ales, France

The effect of biologically active mycotoxin destruxin E, at the dose of 100 µg/ml, on subcellular distribution of several elements (P, S, Cl, Ca, Fe, Ni and Zn) in Malpighian tubules of *Galleria mellonella* larvae was studied *in situ* on ultrathin sections using energy-dispersive X-ray spectroscopy under electron microscope control (electron microprobe). Alterations in elemental concentrations were observed in all compartments and their magnitudes (total of % change of all elements in a given compartment) can be ordered as follows: mitochondria > chromatin > light vesicles > cytoplasm > karyoplasm > dark vesicles. Substantial rise of phosphorus concentration was detected in chromatin and mitochondria after treatment of larvae with destruxin E. The amount of zinc decreased almost by 1/2 in mitochondria.

Electron microprobe analysis thus enables direct *in situ* quantitative evaluation of the effect of mycotoxins at subcellular level on ultrathin sections.

Th-109 IS MILD OXIDATIVE STRESS RESPONSIBLE FOR TERMINAL DIFFERENTIATION OF WI-38 FIBROBLASTS?

G. Saretzka^a, W. Döcke^b, L. Roßner^a and T. von Zglinickia

^aInstitute of Pathology, and ^bInstitute of Medical Immunology, Charité, Humboldt-Universität Berlin, Germany

Cultivation under 40% oxygen for at least four weeks exerts a significant oxidative stress onto WI-38 fibroblasts and blocks the proliferation of these cells completely. In proliferation-inhibited cells, parameters were measured which are known to express characteristic changes during senescence or terminal differentiation, namely lipofuscin accumulation, DNA content, p53 expression, cytoplasmic Na/K ratio and mitochondrial hydration. Results were compared with those obtained from parallel measurements in density-arrested young as well as terminally differentiated populations. In most cases, mild oxidative stress results in effects indistinguishable from those characteristic for "normal" population aging.

Th-111 THE INFLUENCE OF LASER RADIATION ON THE CONTENTS AND DISTRIBUTION OF Ca⁺⁺ IN THE ROOT HAIRS OF PLANTS

K. Skvarko^a, O. Demkiv^b

^a Iv. Franko's University, 290602 Lviv, Univerversitska Street, 1; ^b Istitute of Ecology of Carpathians of the Academy of Sciences of Ukraine, 290000 Lviv, Stefanič Street, 11.

Two kinds of plants were studied: *Lepidium sativum* L. and *Zea mays* L. The lighting of plants (3000 lx), temperature (26°C) and moisture (98%) were controlled during 12 hours of light period in the chamber KTLK-1250 (Germany). The 3-days seedlings, that were cultivated in the medium of Gelrigel were used for experiments. Before germination seeds were radiated with laser light $\lambda = 628,3$ nm, the dose of 0,3-0,6 J without interruption and dose 0,023-0,069 J of scanner's of laser rays under the room temperature. Seeds without radiation were the control group. The relative contents and the distribution of the membrane-binding Ca⁺⁺ dyeing with chlorotetracycline in root hairs were estimated. The intensivity of luminescence was studied cytofluorometrically, by LUMAM-I-ZR (Russia).

It is shown that Ca⁺⁺ in root hairs of these plants are distributed unevenly forming apical-basal gradient. The intensivity of the fluorescence of Ca⁺⁺-chlortetracycline complex in the different points of apical-basal gradient is growing under the influence of helium-neonium laser radiation. Activity inside cell metabolism is discussed. Possibly, the influence of laser radiation realises by means of the phytochrome system of reactions of red-far red light.

Th-110 CHANGES OF PEROXIDASE ISOFORMS CONTENT OF ZEA MAYS LEAVES UNDER INFLUENCE OF CHRONIC IRRADIATION

Prokhnevsky A.I., Sergeeva A.Y., and Sorochinsky B.V. Department of Biophysics and Radiobiology, Institute of Cell Biology and Genetic Engineering, Ukrainian Academy of Sciences, Zabolotnogo St. 148, Kiev, 252143, Ukraine.

Chronic irradiation is known to inhibit plant immunity. Because peroxidase system play an important role in the immunity reaction we have investigated the peroxidase isoforms from control and irradiated plant leaves. *Zea mays* plants were grown at the different lots of the 10-km Chornobyl region. Maximal absorbed dose for plants during vegetation period was 0.79 Gy. Proteins from plant tissues were separated electrophoretically and isoferment quantity was determined with benzidine staining. It was shown that isoferment content from control and experimental tissues was different as well as the total proteins spectrum was different too. We suppose that low levels of chronic irradiation can damage transcriptional processes and cause proteins modifications in the irradiated cells.

Th-112 RESPONSE OF MURINE ERYTHROLEUKEMIA CELLS TO THE ANTICANCER DRUG TAXOL

S. Majumdar, J. Andreassi, M. Metzger, C. Bachman, S. Jones, D. Hickey and K. Reddy
Department of Biology, Lafayette College, Easton, Pa 18042, USA

The anticancer drug taxol, a product of the Pacific yew *Taxus brevifolia*, was evaluated for its effects on multiplication, chromosomes, and the surface ultrastructure of cultured murine erythroleukemia (MEL) cells. The drug stabilizes microtubules by binding to the beta subunit of tubulin. Taxol inhibits cancerous growth by blocking cell division and disrupting cell architecture and has been found to be cytotoxic towards ovarian, breast, lung and melanoma cancers. The MEL cells (clone GM-86) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and maintained at 37°C in a humidified incubator at a 7.5% carbon dioxide level.

Concentrations between 0.1-10 microgram/mliliter of taxol were used as treatment dosages at varying time intervals of 12-120 hours. Trypan blue exclusion and methylene blue assays demonstrated taxol's inhibitory effect on MEL cells proliferation and viability. A cytotoxicity study employing the tetrazolium dye spectrophotometric assay (MTT) showed a dose and time dependent decrease of taxol treated cell viability compared to controls. Mitotic index studies demonstrated taxol's ability to arrest cells in the mitotic phases and thus disrupted the cell division cycle. Taxol altered chromosome numbers, induced chromosomal structural abnormalities, produced micronuclei and increased the frequency of multinucleated cells. The agent inhibited colony formation and caused lysis of MEL cells in the soft agar cloning assay. A scanning electron microscope study revealed that taxol affected surface ultrastructure morphology causing minute holes and pronounced blebbing.

**DETERMINATION OF STRESS PROTEINS IN
WHEAT ROOTS INDUCED BY SALT AND
Th-113 HEAT SHOCK.**

I.Lysova, A.Kostyuk, A.Ostaplyuk.

Institute of Plant Physiology & Genetics, Ukrainian Acad. Sci.,
Kiev, Ukraine.

Synthesis of 110, 105, 98 kD stress proteins was induced by 0.4 M NaCl in roots of cv. Myronovskay 808 wheat seedlings.

In 24 hours after salt action two stress proteins 115 and 110 kD were synthesized. Heat shock (40°C, 24 hours) induced synthesis of heat shock proteins (HSP) 82; 70; 59; 47; 22; 21; 18 kD in wheat seedling roots. First of all the synthesis of HSP 82 kD was induced. In 6-8 hours synthesis of HSP 59 and 47 kD was noticed. Low molecular weight HSP 22; 21; 18kD were synthesized at Lates (12-24 hours).

There are two differences between HSPs and salt induced stress proteins in wheat seedlings roots. Salt stress induced early proteins with higher molecular weights than they for HSPs. Special characteristic of salt stress is the induction of 115 and 110 kD stress proteins in 24 hours after action of salt.

Similar aftereffect of another kinds of stresses (anoxia, heat shock, heavy metals) on induction of these special proteins was absent.

**POLYPEPTIDE COMPOSITION OF BARLEY
ROOT PROTEINS UNDER NaCl AND
Th-114 Na₂SO₄ SALINITY**

A.Kostyuk, I.Lysova, A.Ostaplyuk

Institute of Plant Physiology & Genetics, Ukraine Acad. Sci.,
Kiev, Ukraine.

Polypeptide composition of barley seedling's root proteins under NaCl and Na₂SO₄ have been studied. 0.4 M NaCl salinity induced synthesis of 10 new polypeptides 150; 140; 130; 95; 84; 76; 45; 28.5; 22; 17.5 kD in salt sensitive cv. Nutuns 518. Synthesis of 12 new polypeptides 134; 130; 110.3; 98; 95; 55; 42; 31.8; 28.5; 17; 13.6 kD was induced at this salinity level in salt tolerant cv. Odessky 82. 0.8 M Na₂SO₄ induces synthesis of 6 new polypeptides 76; 54; 49; 47; 28.5; 22 kD in cv. Nutans 518 and 14 new polypeptides 130; 125; 98; 95; 90; 55; 51; 47; 40; 29; 28.5; 16; 15; 13 kD in cv. Odessky 82.

Special feature of salt tolerant cv. Odessky 82 is a synthesis of unique group of high molecular weight polypeptides 130; 125; 98; 95; 90 kD in response to Na₂SO₄ salicene comparing to salt sensitive cultivar. These differences between salt tolerant and salt sensitive genotype of barley are probably connected with specificity of cv. Odessky 82.

**AUTOPHOSPHORYLATION OF GRP94 AND ITS
REGULATION IN DIABETES**
Th-115

P. Csermely

Institute of Biochemistry I., Semmelweis University,
Budapest, Hungary

The 94 kDa glucose-regulated protein (grp94) is an abundant member of the 90 kDa molecular chaperone family in the endoplasmic reticulum and in the Golgi apparatus. grp94 is induced after glucose starvation, was shown to bind the immunoglobulin heavy chain and its possible involvement in chaperoning of secretory proteins was also suggested.

Extending our earlier studies which identified ATP binding site(s) and autoprophosphorylation as intrinsic functions of hsp90, we found that grp94 is also able to phosphorylate itself on Ser/Thr residues. Autophosphorylation of grp94 is activated by micromolar calcium concentrations, has an extreme heat stability, and can utilize both ATP and GTP with relatively high k_m values of 243 +/- 14 uM and 116 +/- 23 uM, respectively.

Analyzing the Western blots of rat liver postmitochondrial supernatants with a monoclonal antibody against grp94 [recognizing an epitope in the region between amino acids 280 and 310 in the protein (antibody 9G10, Biochemistry 23, 4427)] a significant decrease of immunodetectable grp94 could be observed in diabetic rats. This decrease was parallel with a decrease in the Ca-dependent phosphorylation of a 94 kDa protein co-migrating with grp94. All the above effects in diabetic animals were partially or fully reversed by insulin treatment. On the contrary, the overall level of grp94 did not change significantly in diabetic rats as assessed by a polyclonal antibody against the whole protein.

Our observations may reflect a diabetes-induced change in the region 280-310 of grp94 affecting the autophosphorylation of the protein. Autophosphorylation may participate/regulate the complex formation of grp94, and may be involved in its chaperone function.

***In vivo* ³¹P NMR studies of the Antarctic
Th-116 green alga *Prasiola crispa* subjected to
desiccation stress**

C. Bock¹, A. Jacob², A. Mayer¹ and G.O. Kirst²

¹ Inst. of Experimental Physics and ² Inst. of Marine Botany,
University of Bremen, Bremen, Germany

The energy status of *Prasiola crispa* during and after desiccation stress was investigated and the phosphate metabolism was monitored by *in vivo* ³¹P NMR. The effect of desiccation was simulated by successive addition of the nonionic osmoticum PEG-200 (polyethylene glycol). Photosynthesis and respiration were effectively inhibited under these conditions. ³¹P NMR spectra varied in the intensity of Pi, polyphosphates and NTP, although the cytoplasmic pH remained almost constant during stress. The most notable changes were an increase in the cytoplasmic inorganic phosphate signal after desiccation shock, a decrease in and an upfield shift of the terminal polyphosphate signal followed by an appearance of extracellular inorganic phosphate. After return to control conditions the photosynthesis and respiration recovered within four hours as did the concentration of the phosphorous metabolites. An yet unassigned phosphate signal increased in the phosphodiester region of the NMR spectra. Simultaneously the polyphosphate signal recovered in intensity and shift. It is suggested that the decomplexation of divalent cations from phosphate compounds and the phosphate metabolism plays an important role in the distinct desiccation tolerance of *P. crispa*.

TUMOR LIPOPROTEINS AS PROTECTORS OF HYPOXIC TUMOR CELLS AGAINST OXIDATIVE STRESS

Th-117P. Shvartsburg^a. V. Lankin^b

^aInst. Theoret. Exptl. Biophys., Russian Acad. Sci., 142292 Pushchino; ^bCardiological Res. Centre, Russian Acad. Med. Sci., 121552, Moscow; Russia

By the development in vivo the chronically hypoxia, hypoglycemia state in the ascitic tumors, their intracellular lipoproteins (LP) to gain the low resistance for free radicals (as determined by increase the level of peroxidative products-PP). In this case other cell compartments (cytoplasma, nucleous) to conserve its resistance against oxidative stress. That type of cell PP-distribution was not set in exponentially tumor cells, where lipoproteins to showed the high resistance to PP-formation. The loss of the LP-resistance during the growth of tumor hypoxia in vivo is strictly correlated with some important tumor-host metabolic interactions: 1) the intensive transport in vivo the lipids with high portion of polyunsaturated fatty acid (PUFA) from blood and ascitic fluids to hypoxic ascitic cells in which the lipids are primary accumulated in LP; 2) the tendency to decrease the α -tocopherol level in these tumor cells by hold their enzymatic antioxidative activity for glytathione peroxidase, superoxide dismutase. The great distinctions of resistance between LP and other cell compartments, on retention of whole hypoxic tumor cells to oxidative stress led us to propose that PUFA-rich LP can acts as traps of free radicals and prevent their interaction with PUFA-membrane, thus protecting hypoxic tumor cells from lethal oxygen-toxicity.

THE EFFECT OF PENTOXYPHYLINE ON THE ULTRASTRUCTURAL PICTURE OF CYTOPLASMIC MEMBRANE AND ADHERENCE OF CELLS ISOLATED BY BAL-METHOD FROM RATS WITH EXPERIMENTAL INDUCED PULMONARY EMPHYSEMA

Th-119

Sulkowska M., Sobaniec-Lotowska M., Dzięcioł J., Sulkowski St., Kozielcz Z., Famulski W.
Department of Pathological Anatomy, Medical Academy, Białystok, Poland

Ultrastructural changes in cytoplasmic membrane of cells isolated from the lungs of Wistar rats with acute damage of the pulmonary tissue induced by intratracheal infusion of papain solution (2 mg/100 g b.w.) were studied. At the same time the effect of pentoxiphyline upon the pulmonary tissue damage degree was evaluated, as well as upon the composition, adherence and ultrastructural picture of cells isolated from the rat lungs following papain administration.

Damage to the pulmonary tissue, in the histological picture manifesting itself in intraalveolar hemorrhages, was estimated by measuring hemoglobin content in the fluid obtained from multiple bronchoalveolar lavages (BAL). Hemoglobin concentration was determined spectrophotometrically, at wave length 414 nm. The cells were isolated from the lungs after 2 hours following intratracheal injection of papain solution. Adherence degree of cells isolated by BAL method was evaluated by means of the method of McGregor and associates, using nylon fibre as a substrate. The cells undergoing adherence were subjected to the ultrastructural analysis in SEM.

Pentoxiphyline in a dosage applied (10 mg/100 g b.w.), given threefold (1h and 3hs before intratracheal papain administration and just after it) caused a drop in the total number of cells isolated from the lungs by BAL method, particularly in the percentage of neutrophils and in the number of cells undergoing adherence. Moreover, a reduction in the intensity of hemorrhagic changes was observed. The structural analysis of cells in SEM revealed changes in the configuration of cytoplasmic membrane surface which prove the normalization of their functional state.

The results obtained indicate a protective effect of pentoxiphyline in the early period of the pulmonary tissue damage induced by a single intratracheal papain injection.

DNA-PROTEIN METABOLISM IN ATRIAL CARDIOMYOCYTES AFTER NEPHRADRENAL-ECTOMY IN RATS

Th-118G. Selivanova, T. Vlasova,
V. Khirmanov, A. Krutikov

Institute of Cytology RAN, St.Petersburg, Russia

A study has been carried out on left atrial cardiomyocytes of rats with experimental arterial hypertension of the adrenalregeneration type. It was found that the level of polyploidy in the nuclei of left atrial cardiomyocytes 6 and 9 weeks after nephradrenalectomy was the same as that in intact animals. There was an increase in the number of tetra- and octaploid nuclei after 12 weeks. The mean volume of the nuclei decreased by the 9 week followed by a rise, however without reaching the control level by the 12th week. The cytoplasm volume decreased by the 6th week with a further increase on the 9th week and was below the control level by the 12th week. The total protein content in atrial cardiomyocytes also showed a tendency to decrease by the 6th week but was recovered more rapidly in comparison with the volume, reaching the normal level by the 9th week and exceeding it by the 12th week. A conclusion is drawn that prolonged hyperfunction of atrial cardiomyocytes associated with arterial hypertension evokes changes in these cells which manifest themselves in increased polyploidy and protein mass accretion.

LEAD IN THE ULTRASTRUCTURE OF PLANT CELLS

Th-120

A.Woźny, J.Gzyl, K.Idzikowska,
S.Samardakiewicz and
M.Krzesiowska
University of Poznań,
Faculty of Biology, POLAND

The aim of the study was to establish the sequence of symptoms associated with Pb presence in plant cells. As a material for this purpose, species belonging to various taxonomic groups were used.

Early events of Pb presence in the cell included the occurrence of Pb deposits in the apoplast (cell walls, intercellular spaces), the biosynthesis of callose and/or some proteins (e.g. of the superoxide dismutase) as well as changes in the permeability of the plasma membrane. After some time, Pb deposits were observed in otherwise morphologically intact cell structures. For instance, the element was generally observed in ER, perinuclear space, the dictyosomal cisternae and vesicles, the vacuoles, plasmodesmata and cell walls (except for the lignified ones). Much less frequently Pb was found in the nucleoplasm, nucleolus, mitochondrial envelope and thylakoids. Besides the presence of Pb deposits, further symptoms included changes in the structure of the organelles. Among those, the most commonly observed were the concentric configurations of ER and, although less frequently, changes in the structure of the Golgi complexes as well as of the mitochondria. Further changes triggered by Pb presence were similar in character to those associated with the accelerated ageing of cells. Finally, exceeding certain lead content level (different for different species) always resulted in the death of the cells.

STRESS INDUCES VIMENTIN EXPRESSION IN MPC-11 MOUSE PLASMACYTOMA CELLS
Th-121 Guenter Giese^a, Siegbert Holtermueller^b, Andreas Rauscher^a and Peter Traub^a
^aMax Planck Institute for Cell Biology, D-68526 Ladenburg, F.R.G.; ^bCarl Zeiss A.G., D-73447 Oberkochen, F.R.G.

MPC-11 mouse plasmacytoma cells lacking significant amounts of cytoplasmic intermediate filament (IF) proteins can be induced to synthesize and accumulate substantial quantities of the IF protein vimentin by agents like the tumor promoters 12-O-tetradecanoylphorbol-13-acetate (TPA); Giese and Traub (1986), Eur. J. Cell Biol. 40: 266-274) or okadaic acid (Giese et al, manuscript submitted). During the course of establishing conditions for optimum transfection of MPC-11 cells, we found that different transfection protocols by themselves were able to elicit vimentin expression in these cells. For instance, electroporation as well as application of DEAE-dextran and/or chloroquine according to standard transfection protocols induced this expression. Likewise, different agents known to elicit stress responses in different cell types were found to induce expression and accumulation of vimentin in MPC-11 cells. Strong vimentin expression was achieved, e.g., by a short treatment of MPC-11 cells with sodium arsenite followed by cultivation of the cells in conditioned medium lacking this agent, or by prolonged treatment with zinc chloride, while heat stress proved to be a weaker inducer of vimentin expression. We suggest that in certain mammalian cell types vimentin expression may be part of the cellular response to stress conditions. Studies concerning the influence of different stress-inducing agents on vimentin expression are now under way.

MODULATION OF ACTIN FILAMENT DYNAMICS BY HSP27 PHOSPHORYLATION. ROLE IN CELLULAR PROTECTION AGAINST STRESS
Th-122 J.N. Lavoie and J. Landry
Centre de recherche en Cancérologie de l'Université Laval, Québec, Canada G1R 2J6

Heat shock protein 27 is rapidly phosphorylated upon mitogenic stimulation and also during heat shock or exposures to toxic agents. This suggests a common function of HSP27 during early G1 and stress. By comparing the phenotype of Chinese hamster cells expressing either the wild type human HSP27 or a non-phosphorylatable mutant form of HSP27, we demonstrated that HSP27 is a component of a signal transduction pathway that can regulate F-actin polymerization. Overexpression of HSP27 caused an increased concentration of F-actin at the cell cortex and elevated pinocytotic activity. In contrast, expression of the mutant form of HSP27 reduced cortical F-actin concentration and decreased pinocytotic activity relative to control cells. During mitogenic stimulation, wild type HSP27 enhanced induction of F-actin accumulation, whereas mutant HSP27 exerted a dominant negative effect and inhibited the response to growth factors. Similarly to its role during mitogenic stimulation, HSP27 phosphorylation appears to be involved in signaling the re-polymerization of actin filaments that are disrupted during stress. After exposure to cytochalasin D, wild type HSP27 accelerated the reappearance of submembranous F-actin and increased cell survival, whereas the mutant HSP27 delayed the repolymerization burst and failed to induce protection. A similar effect of HSP27 was observed during heat shock or treatment with H₂O₂. The contrasting effect of HSP27 and mutant HSP27 could not be explained by a modification in the intracellular localization of the protein to lamellipodia and membrane ruffles. Interestingly, however, induction of HSP27 phosphorylation causes a modification in the oligomeric size of the protein. Analyses of the signal transduction pathways responsible for HSP27 phosphorylation suggest that HSP27 may be part of a common feedback protective response mechanism activated by a variety of chemical and physical stressors. Supported by the *Conseil de recherches médicales du Canada*.

Degradation of hydrogen peroxide by glutathione peroxidase and catalase in FRTL-5 cells.
Th-123 U. Björkman, R. Ekholm - Department of Anatomy and Cell Biology, Medicinaregatan 3, S-413 90, Göteborg, Sweden

Hydrogen peroxide is formed under normal conditions in all cell types as by-products in electron transfer reactions. In the thyroid H₂O₂ is also generated in a specific reaction and plays a key role in thyroid hormone synthesis. To dissipate H₂O₂ cells possess defense systems which comprise glutathione peroxidase (GPX) and catalase (CAT). We have studied the capacity and mechanisms of H₂O₂ scavenging in the FRTL-5 rat thyroid cell line. The capacity of the cells to degrade H₂O₂ was determined by following the decreasing fluorescence (homovanillic acid) in the culture medium to which a known amount of H₂O₂ (10 µM - 10 mM) had been added. The H₂O₂ degradation rate increased with increasing concentration of H₂O₂ and about 5 % of the added amount of H₂O₂ was degraded per min and 50 µg DNA. Aminotriazole, a specific inhibitor of catalase, did not affect the H₂O₂ degradation rate when the H₂O₂ concentration was 100 µM or lower but had an inhibitory effect at higher concentrations of H₂O₂. The GPX activity (measured as oxidation of NADPH) was about 200 nmol NADPH oxidized / min. mg DNA and was higher in cells cultured in the presence of thyroid stimulating hormone (TSH) than in cultures lacking TSH. Variation of the selenite supply had a clear effect on the GPX activity. A low selenite concentration (obtained by lowering the serum content in the cultures from 5 % to 0.5 %) was accompanied by a decline of the enzyme activity and addition of selenite (0.1 or 1 µM) resulted in a substantial elevation of the GPX activity. This elevation was obtained faster in cells cultured in the presence of TSH than in cultures lacking TSH. In conclusion, FRTL-5 cells possess a high capacity to degrade H₂O₂ and most of this scavenging function can be ascribed to the glutathion cycle but catalase is also involved, especially at high H₂O₂ concentration. The GPX is dependent on selenium and regulated by TSH.

HEAT STRESS RESPONSE OF CYTOSOLIC ADENYLYLATE KINASE IN TOBACCO
Th-124 Uwe Schlattner^a, Edgar Wagner^b, Hubert Greppin^a and Marc Bonzona^a

^a Laboratoire de Biochimie et Physiologie végétales, Université de Genève, 3 pl. de l'Université, CH-1211 Genève 4, Switzerland; ^b Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg i. Br., Germany

In tobacco tissue cultures, total and specific adenylate kinase (AK) activity, protein content and fresh weight decline upon heat stress. Under prolonged heat treatment, these parameters remain at a low level, while they rapidly increase when temperature is reversed to normal. Despite the heat-induced decrease in total AK activity, the activity of the cytosolic isoform increases rapidly (nearly 3-fold within 0.5 h). The increase is transient and hardly detectable after 20 h. It is independent on the time course of total AK activity and does not require a prolonged heat treatment. Cytoplasmic AK behaves therefore as a typical heat shock protein. It is proposed to play a major role in the reorganisation of the energy metabolism upon heat shock. This hypothesis is supported by the well-known heat-shock related response of some glycolytic enzymes and the inhibition of photophosphorylation at high temperatures.

Th-125

AMPLIFICATION OF MDR GENES AND
PROGRESSIVE KARYOTYPICAL
DESTABILIZATION IN CHINESE HAMSTER V-79
RJK CELLS RESISTANT TO ETHIDIUM BROMIDE

T.Grinchuk, L.Lipskaya, E.Sorokina,
I.Artzybasheva, Ju.Panshina, T.Ignatova
Institute of Cytology of Russian Academy of
Sciences, St.Petersburg, Russia

The CHL V-79 RJK cells with different levels of resistance to ethidium bromide (EB) have been studied. Resistant cells are characterized by amplification and overexpression of mdr genes. It is likely that the amplified copies were located at or near the sites of resident mdr gene localization to look as abnormal chromosomal banding pattern (HSRs) in 1q26 loci.

During the selection of resistant cells for increased selective agent concentration or long-term cultivation of the cells under selective conditions progressive destabilization of karyotype was observed. Some new HSRs and specific marker chromosomes were found. The cells have been keeping the degree of amplification, however mdr genes expression was increased by many times.

It's suggested that karyotypical destabilization of the EB resistant cells is due to the amplification of mdr genes.

Th-126

BINDING OF NERVE AND EPIDERMAL GROWTH
FACTORS TO PC12 CELLS: EFFECTS OF
DRUGS AND HEAT SHOCK

V.Buravsky, V.Lukashevich, R.Grons-
kaya, V.Kalunov

Laboratory of Growth Factors and Peptides,
Institute of Physiology, 220725 Minsk, Belarus

Nerve growth factor (NGF) is known to induce differentiation while epidermal growth factor (EGF) stimulates proliferation of rat pheochromocytoma PC12 cells. Binding of their molecules to cell surface receptors precedes and, so limits all other events. To clear up how it depends on membrane microenvironment and the state of cytoskeleton structures we studied effects of phospholipase inhibitors, cytoskeletal active drugs and heat shock on the uptake of NGF and EGF by PC12 cells.

It was found that binding of both factors was decreased at least 2-fold by quinacrine 500 μ M and slightly increased by neomycin 5mM, indicating the significance of phospholipid metabolism in maintenance of certain receptor levels. Treatment of cells with cytochalasin B failed to elicit any changes while colchicine 5 mM reduced the uptake of NGF but not EGF. Heat shock affected differently the receptor binding of the factors. EGF uptake was down-regulated to 30% whereas NGF uptake remained near control levels at 43°C, 45 min.

Thus, the results indicate that receptors for NGF, a differentiating growth factor, more closely interact with cytoskeletal structures than receptors for EGF, a well-established mitogen.

THE EFFECT OF CHELATING AGENTS ON
METABOLISM OF Ni IN THE LIVER

Th-127

J. Koutenský^a, J. Reischig^a, V.
Eyblová^b, M. Koutenská^a, M. Korabecná^a,
P. Vohradská^a, A. Němcová^a, O.
Benádová^b and Z. Žížka^b

^aFaculty of Medicine, Charles University in
Pilsen, Czech Republic, ^bInstitute of
Microbiology, Academy of Sciences, Prague, Czech
Republic

On the one hand, nickel plays an important biological role as an essential trace element and, on the other hand, its compounds exert toxic effects upon the living organism (i.e., carcinogenic, embryotoxic, and cytotoxic effect, induced metabolic changes, influencing some enzymatic system, etc.)

Chelating agents are likely to prevent some injuries that otherwise Ni would cause in an organism, and usually can also change significantly the movement of this metal within tissues.

The aim of our work was to verify whether chelating agents are capable of preventing the toxic effect of Ni on the cells of liver tissue. For this purpose we have used, besides the current EM methods, also the X-ray microanalysis in a transmission electron microscope.

In this study we are comparing both the newly found out results and the newly applied methodical procedures with those ones given in our previous papers dealing with our preceding findings.

Th-128

EFFECTS OF HIGH TEMPERATURE ON CYTO-
MORPHOGENESIS AND ULTRASTRUCTURE OF
MICRASTERIAS DENTICULATA BRÉB.

D. Lehner and U. Meindl

University of Salzburg, Institute for Plant Physiology, A-5020
SALZBURG, Austria

Exposure of growing *Micrasterias* cells (Desmidaceae) to high (30°-38°C) temperatures produces changes in morphology that are accompanied by several ultrastructural alterations. Up to 30°C cell shape develops normally. Rising the temperature for a few degrees leads to a reduction of size and pattern of the young half cell even when the treatment is limited to 5 minutes. The most striking effects occur during longer treatment (60 - 90 min.) with temperatures between 33° and 36°C and include a loss of the cell symmetry and an increase of "main lobes" with reduced degree of differentiation. At 38°C cell growth is completely inhibited even if the treatment lasts for only 5 minutes.

The main ultrastructural alterations are an increase in amount and length of ER cisternae, a reduced number of ribosomes and polysomes, the appearance of "heat shock granule" aggregations localized in the cytoplasm, dictyosomes with an increased number of cisternae and a much thicker primary wall with unusual vesicle-like inclusions.

It is assumed that temperature effects on morphogenesis and ultrastructure of *Micrasterias* are mainly caused by alterations of membrane lipids and protein synthesis.

Th-129 GERM AND SOMATIC CELL RESPONSE TO PHEROMONAL STRESSOR IN HOUSE MICE;
E. Daev, O. Matzkewich, E. Antonjuk
Laboratory of Animal Genetics, Biological Institute of St. Petersburg State University, St. Petersburg, 199034, Russia.

It is well known that pheromones of some species of rodents can act as a stress-factors. They are highly active volatile substances playing an important role in the control of reproduction. In mice pheromones of mature males disturb hormonal state, induce adrenal hypertrophy and delay puberty of young males.

Young mouse CBA males were exposed for 2 hrs to volatile urine substances (VUS) of adult males of the same strain. Germ cells at metaphase I stage and bone marrow cells at anaphase-telophase stage were analysed 24 hrs after exposure. Frequencies of different meiotic and mitotic disturbances were revealed.

It is shown that the level of meiotic disturbances (multivalent associations, autosomal univalents and fragments) at metaphase I stage is significantly higher (more than 2 times) than in control animals. Cytogenetic analysis of bone marrow cells at anaphase-telophase shows that there is a significant increase of bridges, fragments and delayed chromosome levels in young VUS-treated males.

Possible role and mechanisms of the cytogenetic response to pheromonal stressors (and some other specific pheromonal effects obtained (Tzapigina, Daev, Aref'ev, 1991)) are discussed.

Th-131 MITOTIC ACTIVITY IN ROOT TIP OF ALLIUM CEPA L. TREATED BY VANADIUM
H. Zaporowska, M. Slotwińska

Department of Cell Biology, University of M. Curie-Skłodowska, Akademicka 19, 20-033 Lublin, Poland

Vanadate at nanomolar to micromolar concentrations stimulates DNA synthesis, proliferation and differentiation of animal cells. The actions of vanadium on plant cell proliferation and metabolism are much less studied.

The aim of the present study was to examine the influence of different doses of vanadium on plant cell division.

The roots of Allium cepa L. were treated with the aqueous solution of ammonium metavanadate - NH₄VO₃/AMV; Reachim, Russia/ at concentrations 50, 250, 500 and 1000 μM for the period 1 and 2 weeks.

AMV at concentration 50 μM have not effect on the root growth and mitotic activity of root meristem cells during first week of investigations. AMV at concentration 250 μM inhibited the mitosis. Whereas, AMV at concentrations 500 and 1000 μM inhibited completely the mitosis after 4-5 days. The inhibition of mitosis was dependent on concentration of AMV and time of incubation.

The investigations showed also the morphological changes in cells, particularly vacuolization of cytoplasm in cells treated with AMV solution of highest concentration.

Th-130 MORPHOHISTOCHEMISTRY OF THE MATRIX OF DEGENERATED LUMBAR DISC CARTILAGE
E. Dema, M. Dragan, S. Sarb and L. Vasile
Department of Histology, Medicine University, Timisoara, Romania

We researched the aspects of the matricial-cells on the bioptic fragments of lumbar intervertebral discs. The study was done on 12 cases (2 females and 10 males) aged between 22-65 with discal suffering, by usual morphological methods and few histochemical reactions for praising of glycoproteic and glycogenic substances (PAS reaction and salivary amylase-PAS reaction), glycosaminoglycanic substances (toluidine blue at pH 5 and pH 2.8 and Alcian blue at pH 2.8 and 0.5 and combination Alcian blue-PAS). All the cases were clinically and paraclinically investigated, the 22 patients being operated for lumbar disc degeneration. After fixation in 10 % solution of formalin or Bouin's fluid and decalcification with 10 % trichloracetic acid chemically pure, the fragments were stained with H and E and histochemical stainings: toluidine blue, Alcian blue, Alcian blue-PAS. We could observe different cells degradative changes (nuclear and cytoplasmatic changes), chondrocytes vacuolisations and matrical desorganisation.

Special histochemical tissue reactions were seen: the matrical metachromasia to toluidine blue at pH 2.8, Alcian blue positivity at pH 0.5 and PAS positivity which resisted at salivary amylase digestion of the matrical quasinormal areas.

Th-132 ULTRASTRUCTURE OF NERVE AND GLIAL CELLS IN SPINAL GANGLIA OF HIBERNATING ANIMALS

D. Krajčí
Department of Anatomy and Electron Microscopy Microscopy Unit, Faculty of Medicine, Kuwait University, KUWAIT, State of Kuwait, and Department of Histology and Embryology, Faculty of Medicine, Palacky University, OLOMOUC, Czech Republic

In order to verify the significance of our previous investigations of spinal ganglia in hibernating animals the same ganglia of bats were collected during the winter period of their circannual life cycle and processed for the light and electron microscopy examinations.

Although the overall structure of nerve and glial cells was found normal compared to the same in active animals, the cytoplasm of some nerve cells displayed distinct alterations in the ultrastructure of their granular endoplasmic reticulum regarding its pattern and relation to ribosomes. Several spots of stacked and smoothed ER membranes were found wrapped up into concentric shapes resembling autophagic vacuoles. Free ribosomes accumulated in the peripheral cytoplasm, and cisternal portions Golgi complexes were reduced in favour of their vesicular components.

Neuroglial interrelations were found less altered as was also the ultrastructure of satellite cells' cytoplasm. These recent observations are discussed and related to our previous investigations of the same organs in hibernating hedgehogs and hypothermic frogs.

Th-133 NITRIC OXIDE MODULATES POSTHYPOTIC STRESS PROTEIN RESPONSE IN THE GUT
B. Gebhardt, J. Stein, J. Ries, W.F. Caspary.

2nd Department of Medicine J.W. Goethe-
 University, Frankfurt, Germany

In previous studies we demonstrated that intracellular reperfusion damage is represented by the synthesis of heat shock proteins (HSP). HSP induction, therefore, is triggered by a superoxide radical (O_2^-)-dependent protein damage. In this study we investigated the influence of nitric oxide on hypoxia/reperfusion injury and posthypoxic stress protein induction, focussing on the major heat shock protein, HSP72. As an *in vitro* model the small intestinal crypt cell line IEC-6 was used. **Methods:** In hypoxia the medium from IEC-6 cells was replaced by oxygen-free Ringer buffer and cells were perfused with nitrogen in an air-tight chamber for various times. HSP synthesis was detected by radioactive protein labelling after reperfusion and two-dimensional gel electrophoresis or immunological identification. To determine lipid peroxidation during reoxygenation (LDH)-release was measured photometrically. O_2^- was detected by MTT/formazan blue reduction and measured quantitatively. To determine the influence of nitric oxide (NO) we used the inhibitor of NO-synthetase NG-nitro L-arginine methyl ester (L-NAME) and sodium nitroprusside as a NO liberating substance. **Results:** Stress proteins were detected after 2h of hypoxia and showed an increase in synthesis under the influence of nitroprusside, while L-NAME decreased the stress response. All substances had no effect under control conditions. The xanthine oxidase-dependent O_2^- production was significantly increased after 1h of hypoxia ($p < 0.005$) increasing furthermore after 2h and 3h. L-NAME decreased the formazan blue production significantly ($p < 0.05$) after ischaemia and under normal culture conditions ($p < 0.05$). NO liberation by nitroprusside enhanced the posthypoxic formazan blue formation significantly, having no effect in controls. LDH release increased in reperfusion and showed significance after 1h (212 mIU/10⁶ cells; $p < 0.005$) and after 2h and 3h ($p < 0.05$). L-NAME had no significant effect on LDH release, while sodium nitroprusside showed a mild protection. We conclude that NO influences the O_2^- -induced protein damage that induces stress protein synthesis, but is protective against membrane damage. O_2^- , modulated by NO, seems to have a central role in this process, but it is not clear whether metabolites or O_2^- caused the damage. The protein-injuring effect of NO, therefore, contradicts the commonly presented postischaemic NO protection.

Th-134 HEAT-SHOCK ALTERS THE ACTIVITY OF NUCLEAR PROTEIN KINASE C OF LYMPHOID TUMOR CELLS
Szabó, K. and Hidvégi, E.J.

Natl.Res.Inst.Radiobiology and Radiohygiene, Budapest 1775, Hungary

Hyperthermia, i.e. exposure of tumor cells to slightly elevated temperature, is being used successfully in therapy of certain cancers. In the present study P388 lymphoid leukemia tumor cells were treated at 43.5°C and 45°C, *in vitro*, for various lengths of time, then nuclear and cytosol fractions were isolated. The activities of various protein kinases were differentiated by using enzyme activating and inhibiting conditions. In the first five minutes hyperthermia resulted in a rapid, transient increase both in the calcium/phospholipid dependent and in the calcium-independent/diglyceride-activated protein kinase C activities of the nucleus. A second prolonged elevation of nuclear protein kinase activity was observed after longer (20-60 min.) hyperthermic treatment. Phosphorylation of some nuclear proteins exhibited different and characteristic patterns during the treatment. Proteins of 15,18,20 kDa and histone H1 seemed to be phosphorylated selectively by the calcium-ion and phospholipid-dependent protein kinase C.

These results suggest that specific phosphorylation of various proteins, directed by isoenzymes of protein kinase C, is involved in the hyperthermia induced physiological changes of tumor cell nucleus.

Th-135 REACTION OF THYMUS BIOGENIC AMINES TO HARD IMMobilIZATION AND LIGHT INJECTION STRESS
Boris M. Gordon Jr.

Department of Histology, Ilya Ulyanov state University,
 Cheboksary, Russian Federation

Great changes in thymus histochemical picture were seen after hard acute 24-hour immobilization stress. First of all, macrophage-looking cells of pre-medullar thymus zone, described before as producer of histamine & monoamines, decreased their number and brightness. The same cells of subcapsular zone, described before as amino-consumers & deponents, increased their number and brightness. Some of myeline nerves began to take histamine & serotonin to their cover, it was good seen after histochemical studying. Amine content in lymphocytes also increased. Maybe cause of increased level of all amines in microenvironment differentiation of thymus mast cells became quicker and their number greatly increased in stroma & parenchyma of thymus, their degree of methachromasia also had become deeper, leading to gamma, that was not described for rat mast cells before. In some cases plasmacyt cells were seen in thymus, that is also not often variant.

Light injection stress did not produce so great changes, but direction was the same. When the presence of antigen in injection took place, direction of reactions changed.

Th-136 PEROXIDATIC ACTIVITY RELEASED IN HEART EFFLUENT AS A BIOCHEMICAL PARAMETER IN THE STUDY OF ISCHEMIA/REPERFUSION INJURY
M.V. Leabu, S. Musat, O.C. Trifan

Department of Cell Biology and Histology,
 "Carol Davila" University of Medicine and Pharmacy, Bucharest,
 Romania

Peroxidatic activity released in heart perfusion/reperfusion buffer was defined as a biochemical parameter in the study of ischemia/reperfusion injury. The level of peroxidatic activity was determined by dot blot analysis with 3,3'-diaminobenzidine as hydrogen donor and hydrogen peroxide as substrate. Before ischemia, the heart effluent showed low level of, or practically leaked peroxidatic activity. After ischemia the level of peroxidatic activity in reperfusion buffer was 2-3 times higher. The modulation of ischemia effects on heart treated with Aprikalim or Nicorandil could accurately be noted using peroxidatic activity as biochemical parameter. The results in control and drug-treated hearts were in good agreement with those showed by other enzymes used as biochemical parameters in the study of ischemia/reperfusion effects on myocardium. The results recommend the peroxidatic activity released in heart effluent as an alternative useful biochemical parameter in the study of ischemia/reperfusion injury on myocardium.

MODEL OF ION-OSMOTIC HOMEOSTASIS FOR A SINGLE CELL UNDER COLD IONIC STRESS CONDITIONS.

Th-137

K.B.Asanidi, D.M.Vachadze
Laboratory for Cellular Biophysics, Institute
of Theoretical and Experimental Biophysics
RAS, Pushchino, Russia.

A mathematical model which enables a quantitative interpretation of the results of electrophysiological, morphometric and bioenergetic measurements for nonexcitable cells during exposure to cold but nonfreezing temperature is proposed.

As the basis was used the Boyle-Conway Model including the Effect of an Electrogenic Pump for Nonexcitable Cell /Aslanidi,Panfilov,1986/. To describe the Na-K-ATPase reaction we used the scheme of simultaneous activation of the enzyme in which the rate of ATP hydrolysis with ionic pump is shown as experimental temperature dependence, known as Arrhenius curve, for different organisms.

Using the equations of the model can be computed dependences of the membrane potential, intracellular sodium, potassium, chloride and calcium ionicactivities, all ionic fluxes and cell volume on temperature.

The model shows the decrease of the potassium activity, the membrane potential, and all ionic fluxes, and increase of the cell volume, sodium, chloride and specially calcium activities accompanying the decrease of temperature. Moreover, the model shows cytosolic calcium disbalance accompanying the decrease of temperature upto the level lower than critical for individual organism promoting to the ionic stress and cell death.

Comparison between model predictions and experimental results is carried out for different clonal lines of mammalian cell culture /Potapova,Vesely,1981/, embryos and eyed eggs of fishes with different cold adaptation.

ON THE PRESENCE OF METALLOTHIONEIN IN EGGS OF VARIOUS SEA URCHIN SPECIES

Th-139 R. Scudiero^b, P.P. De Prisco^a, C. Capasso^a, A. Capasso^a, S. Filosa^b, E. Parisi^a

^aC.N.R. Institute of Protein Biochemistry and Enzymology, Naples, Italy; ^b Department of Evolutive and Comparative Biology, University of Naples, Italy

In many organisms, the resistance to environmental stress by heavy metals is mediated by low molecular weight, metal-binding, cysteine-rich proteins termed metallothioneins (MTs). The MTs present in eggs of six different sea urchin species were isolated by gel permeation chromatography and quantified by silver saturation assay. The two mediterranean species *P. lividus* and *S. granularis* were found to contain large amounts of MT, while no MT was detected in *A. lixula* and in the far-eastern species *S. intermedius*, *T. hardwickii* and *C. japonicus*.

A zinc-binding protein unlike MT was isolated from the last three species; this protein was apparently not present in all the three mediterranean species examined. A cadmium-binding protein was isolated from *A. lixula* embryos grown in the presence of sublethal concentrations of metal. This protein, characterized by PAGE and amino acid composition, showed a low molecular weight and a high cysteine content, thus resembling an MT. These results demonstrate that in some species, eggs and embryos may be devoid of MT, which, however, may be expressed as a response to the presence of heavy metals in the environment.

A Model for Hypoxia and Reoxygenation

Th-138 at the Blood-Brain-Barrier

K.Mertsch, T.Grunz, A.Ladhoff, I.Blasig
Research Institute for Molecular
Pharmacology, A.Kowalekstr.4, 10315 Berlin,
Germany

Radical-induced lipid peroxidation was investigated in comparison to biochemical and morphological alterations in cultures of brain endothelial cells which underwent hypoxia. The content of TBARS was enhanced to 2.50 ± 0.46 nmol/mg protein after hypoxia and 5.92 ± 0.54 nmol/mg protein after reoxygenation compared to normoxic control 1.79 ± 0.21 nmol/mg protein. ATP-content decreased during hypoxia to 7.9 ± 1.6 nmol/mg protein, LDH release increased to 172 ± 62 mU/mg protein (VS. 15.7 ± 3.1 nmol/mg and 90 ± 64 mU/mg). Morphological changes after hypoxia (lysosomes, vacuoles, multivesicular bodies) and especially after reoxygenation (vacuoles, blebs, lipofuscin granula) indicate cell damage (and BBB-damage) mediated via peroxydation of membrane lipids due to formation of free radicals.

INFLUENCE OF LOW OXYGEN PRESSURE EXPOSURE ON LUNG MORPHOLOGY AND LIPID

Th-140 PEROXIDATION PROCESSES

L.V.Mogilnitskaya, V.N.Prakof'ev,
A.A.Khodakova

Institute of Biology, Rostov-on-Don, Russia

Effect of low oxygen pressure exposure (0.3 MPa) during 5 h on white rats lung was studied. Animals were decapitated the moment on completion of oxygen exposure and on days 1, 3 and 7 after exposure. Significant changes in lung morphology were occurred and supported by biochemical investigations in all studied periods.

Oxygen exposure yielded a 49% increase of histamine concentration. Simultaneously, diene conjugates and Schiff's bases increase are caused by 54% superoxide dismutase (SOD) activity reduction as compared with control.

Histamine content raised by 121% on day 1 and normalised on day 3 after oxygen exposure. Lipid peroxidation (LPO) products level continued to elevate till day 3 after oxygen exposure. Only on day 7 their level fell down but didn't reach control value. This was accompanied with low SOD activity. It was by 44-74% below control level on days 1, 3 and 7 following to exposure.

Having a mind a peculiar role of superoxide anion radical in LPO it becomes understandable the accumulation LPO products against the background of SOD inhibition in lung tissue after single 0.3 MPa oxygen exposure during 5h. Thus, oxygen-induced changes of studied biochemical parameters in lung tissue remain for the extended period.

THE RESPONSE OF MAIZE CALLUS TO USTI-LAGO ZEAE (BERK.) CULTURAL FILTRATE
Th-141
Gutsulyak O.P. and Balashova N.N.
 Institute of Genetics, Kishinev, Moldavian Academy of Sciences

The cultural filtrat action was proved on maize calli belonging to 6 genotypes with different levels of resistance to corn smut. The cultural filtrate was added to nutrient medium in various concentrations of 10-60% V/V. The calli were incubated on the medium with the cultural filtrate in three weeks afte callus initiation. When the concentration was 15-20% the callus biomass growth decreased by 10-40% in dependance on a genotype when the concentration of the cultural filtrate was 40% the callus growth stopped completely and it perished in 8-10 days. Therefore studing of the cultural filtrate action was carried out when the concentration amounted to 20-30%. Callus incubated on a medium with the cultural filtrate of 25% showed that the callus biomass growth amounted to 10-20% in the first week and during the second week the resistant lines showed stronger callus growth (40-50%) while the susceptible lines showed only their growth of 10-20%. The cell response was peculair in the fact that in two weeks rhizogenesis began to occur which first developed at a spot of the contact with the medium and then over the whols callus surface. There was a difference of the rhizogenesis intensity between the resistant and susceptible genotypes.

THE ANTER RESPONSE TO THE EFFECT OF FUSARIUM GRAMINEARUM CULTURAL FILTRATE
Th-142
RATE
Fandeeva A.V.
 Institute of Genetics, Kishinev, Moldavian Academy of Sciences

The experiments were carried out to study 5 triticale cultivars with alternative resistance to Fusarium-blight. The anthers were cultured on nutrient media containing the cultural filtrate in the concentration of 0-40%. Insignificant increase in frequencies of callus developing was observed on the nutrient medium containing of the 10% cultural filtrate in comparison with the concentration of 5% that might be due to the presence of substances stimulating callus developing(auxins and others). 20%of the cultural filtrate added to the medium caused significant suppression of the callus development and when the cultural filtrate amounted to 30% the frequency of the callus development was 2-3% from all the planted anthers. The maximal difference between the genotypes for this particular trait was observed when the concentration of th cultural filtrate amounted to 15-20%. Consequently, both phytotoxic and phytohormonal effects of Fusarium graminearum metabolites were established on an ability of triticale anthers to develop callus.

THE ORGAN-SPECIFICITY PATTERN OF ISOZYME SPECTRUM EXPRESSION OF "HOUSEKEEPING" GENE IN CATTLE TISSUES
Th-143

V.I.Glazko, U.E.Bodnaruk, S.I.Tarasuk
 Laboratory for Molecular Genetics of Farm Animals, Institute of Animal Breeding and Genetics v.Chubinskii, Kiev-Borispol, 256319, UKRAINE

The organ specificity of the expressions of number of enzymes of the intracellular metabolism was studied in cattle tissues. The greatest organ-specific variability was detected for esterases, kreatine kinase, hexokinase, adenylate kinase and glucose-6-phosphate dehydrogenase. The liver and heart exhibited the most complexity biochemical phenotypes. Some unique zones of esterases were detected in the ovaries. The heart and kidney esterase spectrum was changed in cows, that maintained in 30 km zone of Chernobyl accident.

MECHANISM OF THE STRESS-PROTECTIVE EFFECT OF DELTA-SLEEP-INDUCING PEPTIDE
Th-144
N.I.Uskova, A.V.Lysenko, A.M.Mendzelevskii, P.E.Povilaite

Neurocybernetics Research Institute, Rostov State University, Russia

Phase amplification of the main inhibitory brain system (GABA-ergic) in DSIP modulatory effect realization both in time and under stress of different duration and activation of axo-somatic synapses III-V layers of cerebral cortex has been found. The majority of these synapses belong to GABA-ergic ones which are responsible for inhibitory processes in cortex. DSIP ability to change the activity of neutral proteinases (protaminases and calpaines) in rat brain and cathepsin D of lysosome fraction without the total protein content changes has been also determined. We assumed that results obtained demonstrate protein circulation acceleration without any displacement to catabolism and does not exclude the possibility of limited proteolytic processes initiation by DSIP.

Thus, prophylactic injection of DSIP may have adaptive effect due to increase of biologically active peptides amount, i.e. main bioregulators which determine metabolism character and normal functioning of central nervous system; and due to DSIP ability to support optimal ratio between inhibitory and excitatory amino acid neuromediators.

MYOCARDIAL ANTIOXIDANT METABOLISM IN
NORMOTENSIVE AND HYPERTENSIVE RATS

Th-145

H. Hong and P. Johnson

Department of Chemistry and College of
Osteopathic Medicine, Ohio University, Athens, Ohio, USA

The levels of lipid peroxidation (LPO) and antioxidant enzyme levels in the myocardium of normotensive (WKY) and spontaneously hypertensive (SHR) adult male rats were analyzed. Comparison of the levels of enzyme activities between SHR and WKY rats shows that SHR rats have higher levels of glutathione peroxidase (GPX) and catalase (CAT) activity in both sedentary and exercised states, and that the level of glutathione reductase (GR) activity is higher in exercised WKY rats. The effect of exercise within each group is to cause similar decreases in the levels of GPX, GR and CAT activities. Although there was a significant increase in the LPO level for the exercised group in WKY rats, the difference between LPO levels in sedentary and exercised SHR rats was not statistically significant. These studies indicate that in comparison to WKY rats, SHR rats show a constitutive increase in the levels of certain antioxidant enzymes (GPX and CAT), and that although these levels are decreased during exercise, the level of oxidative damage in the SHR rats is not increased as it is in WKY rats. This difference may be solely the result of the higher levels of the above-mentioned antioxidant enzymes in the hypertensive animals, or it may also be due in part to changes in other antioxidant enzymes and antioxidant metabolites. (Supported by the American Heart Association, Ohio Affiliate)

THE INFLUENCE OF BUFORMIN BOUND ON POLY-
MERIC SUPPORT IN ALLOXAN-INDUCED DIABETIC
RATS

Th-146

A. Carpov^a, C. Filip^b, G. Mocanu^a, M.
Nechifor^b, A. Negru^c, E. Teslaru^c

^aInst.Macro.Molec.Chem. "P.Poni" Iasi; ^bDept.
Pharmacology, Univ.Med. Pharm. Iasi - Romania

We investigated the influence on the blood glucose levels of free Buformin (B) and Buformin bound on different polymeric supports (BBPS) in experimentally induced diabetic rats.

We worked on 3 series of 6 adult male Wistar rats each, bred in identical laboratory conditions, without food 18 hours before the experiment started.

48 hours before the experiment, all series have received alloxan, 175 mg/Kg b.w., s.c.

Series I served as control, series II received B, 50 mg/Kg b.w., p.o., series III, IV, V, VI received BBPS in equivalent doses with those of B.

The blood glucose levels have been determined by orto-toluidine test at 1, 3, 5, 8 hours after B administration.

The obtained data show a decrease in the blood glucose levels in series III, III, IV, V, VI as compared with control series. Eight hours after B and BBPS adm., the decrease of the blood glucose levels was higher in series IV which received BBPS (SF-Filtradex 0,237 g B/1 g polymeric support) ($1,12 \pm 0,09$) than in series II which received B ($1,33 \pm 0,14$).

STUDY OF HEAT-SHOCK PROTEIN KINETICS OF
EXPRESSION IN THE EUBACTERIUM *Strepto-*
myces coelicolor

Th-147

Jiri Vohradsky^a Anna Maria Puglia^b Charles J.
Thompson^c

^aCzech Academy of Sciences, Inst. of Microbiology, Praha, Czech Republic; ^bDip. di Biologia Cellulare e dello Sviluppo, Palermo, Italy; ^cBiozentrum of University of Basel, Basel, Switzerland.

Quantification of global gene expression by two-dimensional (2D) gel electrophoresis is potentially a very powerful technique to study any eucariotic and procarciotic developmental system. One gel allows the quantification of large numbers of proteins at any one time and a series of gels record overall patterns of change in gene expression which define a process of biological differentiation.

In response to various stress conditions, *Streptomyces* activate antibiotic biosynthesis pathways and undergo morphological differentiation. Our experiments were designed to study heat shock biosynthetic regulons associated with this process. Proteins were radiolabeled at various times during development and in response to heat shock, resolved on 2D gels and then detected and quantified by PD-Quest gel analysis software. Statistical analysis was used to identify gene products exhibiting increased synthesis after exposure of the culture to stress (heat shock). Kinetics of these proteins was recorded as a function of growth and time after exposure to a heat shock. The observed kinetics of expression suggested systems of coordinate control and indicate the moment during development, when these systems were activated.

THE STRUCTURAL-FUNCTIONAL CHANGES IN
CELLS OF THE ADRENAL MEDULLA AFTER
IRRADIATION WITH LOW DOSES

Yu. A. Shostak

Laboratory of Morphology and Cytogenetics,
Institute of Radiobiology, Minsk 220141, Belarus

A study was made of the pattern of changes in structural-functional indices in cells of the adrenal medulla of young and adult albino rats subjected to external whole-body gamma-irradiation with doses of 0,5 and 1,0 Gy (dose-rate of $2,7 \times 10^{-4}$ Gy/s).

It was demonstrated that adrenaline- and noradrenaline-containing cells of the adrenal medulla of young and adult rats suffered the similar morphological changes, expressed in the hypertrophy and destruction of mitochondria, apparatus Golgi and endoplasmic reticulum, violation of integrity of cellular and organelle membranes, vacuolization and hardening of cytoplasm and deformation of nuclei. The changes of some stereological parameters (such as the areas of cells, nuclei, cytoplasm, quantity of catecholamine granules and others), as well as of levels of key enzymes of the tricarboxylic acid cycles (succinate dehydrogenase), glycolysis (lactate dehydrogenase) and catecholamine metabolism (monoamines oxidase) in different periods of observation were mainly of phase nature and indicated the development of adaption syndrome in the animal organism.

HSP70 INTERACTION WITH GAPDH
FROM BOVINE MUSCLES

Th-149 A.Kinev & B.Margulis

Institute of Cytology, Tikhoretski av.
4, St.Peterburg, 194064, Russia

A protein with MW=37kD and pI8,8-9,0 was found to coimmunoprecipitate with hsp70 from bovine muscle extract. After chromatography of the extract on hsp70- Sepharose column, a protein with the same characteristics was a major component retained. The protein was identified as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.2.12) by microsequencing of peptides. The hsp70-GAPDH complexes dissociated in the presence of NAD, but not in ATP-Mg or ADP-Mg. The absorbed enzyme demonstrated specific dehydrogenase activity. In solution purified hsp70 was shown to recover GAPDH activity inhibited beforehand by ADP. Taking in account that: 1) ADP splits GAPDH to dimers; 2) cells contain relatively low content of NAD when compared to that of GAPDH; 3) tetra- and dimers of the enzyme show different activities at low concentrations of NAD, we suggest the role of hsp70 in vivo in a regulation of the enzyme activity on a level of subunit interaction.

ANTIBODY AGAINST ANGIOTENSIN II AT,
RECEPTOR COMPLETELY PREVENTS THE DEVELOP-
MENT OF TWO-KIDNEY, ONE-CLIP RENAL HYPER-
TENSION IN RAT

B. Železná^a, L. Veselsky^a, V. Jonáková^a,
I. Bláha^b, J. Velet^b, J. Zicha^c and
J. Kuneš^c

^aInst. of Molec. Genetics, Praha, ^bInst. of Org. Chem.
and Biochem., Praha, ^cInst. of Physiology, Praha, Czech
Republic

The development of two-kidney, one-clip (2K1C) renal hypertension after the chronic blockade of angiotensin II (Ang II) AT₁ receptors with antibody against the receptor was studied in Wistar rats. The rats were immunized with the short peptide corresponding to the Ang II binding region of the AT₁ receptor. Multiple peptide attached to lysine oligomer was used as an antigen.

Tail-cuff systolic blood pressure was measured every week and direct blood pressure four weeks after the 2K1C constriction. The constriction of renal artery resulted in fast and large increase of blood pressure in non-immunized animals, but in immunized animals the development of renal hypertension was prevented with antibody against the part of AT₁ receptor.

CELL MEMBRANE OLIGOSACCHARIDES(OS) MODULATE ES-
TROGEN RECEPTOR(ER) HETEROGENEITY:MEMBRANE OS
Th-151 SIGNAL GLYCOSYLATION(GLY), WHILE ESTROGENS(E)
SIGNAL PHOSPHORYLATION (PHOS) A.A. Hakim.
Cell. & Mol.Biology. 180 Longwood Drive.PO Box
994. Kankakee. Illinois 60901. USA.

During human epidermal mammary cell (HEMC) mitogenic stimulation & BCa infiltration into the lymph node, stage-dependent structural changes occur in the membrane OS (Hakim PA SEB J 2/4:A3009, 1988; 2nd Int.Conference Met.Res.Soc.Heldelberg Sept 27, C-43, 1988). These changes lead to structural heterogeneity of BCa cell membrane OS (Hakim PASEBM185; 155-176, 1987) accompanied with Gly of BCa-growth factor receptor (BCaGF-R). Similar changes occur in the colon membrane OS (Hakim PASEM Proc 46:1317, A5838, 1987) where the colon cell alterations correlate with cell colonogenicity & metastatic ability. The PCA-soluble serum & tumor cell membrane glycoproteins form an Index of tumor burden (Hakim Cancer Det.Prev.5:201-205, 1983) where the carbohydrate moieties are linked to tyrosine(T), serine(S) & threonine(Th) residues. Estradiol(E) Glycosylate BCaGF-R Phosphorylate PTk. Thus BCa cells receive both Gly & Phos signals directed to T,S & Th residues of the PTks. This study reports on the relationship between Gly/Phos of PTk-R during progressive & aggressive proliferation. Human MCF-7, ZR-75.1 hormone-independent BCa cell lines (Hakim Expt.Cell Biol.54:193-211, 319-332, 986, & PASEBM 185, 158-176, 1987). The ER,EGF-R,TGF-R,PDGF-R, & the oncogene Products P185^{HER/neu}, P60^{src} & P21^{RAS} were determined as in (Hakim J Surg.Oncology 40:21-31, 1989; & Diagnostics 27:30-37, 1989) & monitored in absence & presence of E. Estradiol produced in MCF-7 & ZR75.1 significant increase in Phosphorylated T,S,Th which correlated with increases in P185^{HER/neu}/P21^{RAS} (12 to 15 fold) & P60^{src} (8-10fold). Tamoxifen(Tm) blocked E-effects. In E-responsive BCa cells Phos is more prominent than Gly, while in E-non-responsive BCa cells Gly is more prominent than Phos pathways.

EFFECT OF GALACTOGLUCOMANNAN DERIVED
OLIGOSACCHARIDES ON 2,4-DICHLORO-
PHENOXYACETIC ACID STIMULATED
Th-152 ELONGATION GROWTH OF PEA AND SPRUCE
STEM SEGMENTS

O. Auxtová, D. Lišková, D. Kákoniová, M. Kubačková, Š. Karácsányi and L. Bilisics
Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava, Slovak Republic

Galactoglucomannan derived oligosaccharides [GGMO] obtained from galactoglucomannan of poplar (*Populus monilifera* H.) wood consisted of galactose, glucose and mannose in the mole ratio 0.2 : 1.0 : 2.9 GGMO (d.p. 4-8) were shown as inhibitors of the 2,4-dichlorophenoxyacetic acid (2,4-D) stimulated elongation growth of pea and spruce stem segments. A dependence on the concentration of oligosaccharides (between 10⁻⁵-10⁻¹⁰ M) as well as plant species was ascertained. The pea stem segments were much more sensitive (10⁻¹⁰ M) than spruce (10⁻⁸ M). The oligosaccharides did not exhibit toxicity at all. Time course in long-term bioassays, and timing of oligosaccharides and auxin action in the growth process were also studied.

Th-153 MODULATION OF DIFFERENTIATION ALTERS ADHESIVENESS AND INVASIVENESS OF CHORIOCARCINOMA CELLS

M. Linke, H.-P. Hohn, H.-W. Denker
Institute for Anatomy, University Hospital, D-45122 Essen, Germany

During blastocyst implantation human trophoblastic cells undergo a differentiation program in preparation for the various functions they have: in early pregnancy adhesion to the uterine epithelium, penetration of the basement membrane, and invasion into the endometrial stroma; later on production of pregnancy hormones and embryo nutrition dominate.

We are studying, *in vitro*, how adhesiveness and invasiveness of human trophoblastic cells are correlated with differentiation using choriocarcinoma cell lines (BeWo, JAR, JEG-3) as a model. Cells were treated with modulators of differentiation: retinoic acid (RA), methotrexate (MTX), dibutyryl cyclic AMP (cAMP) and phorbol-12-myristoyl-13-acetate (PMA). Differentiation was monitored by measuring secretion of human chorionic gonadotropin (hCG). Two aspects of adhesiveness were tested: (i) cell-cell-adhesion of multicellular choriocarcinoma spheroids to monolayers of an uterine epithelial cell line (RL95-2); (ii) cell-matrix-adhesion of single cells to components of the extracellular matrix (ECM). Invasiveness was measured in a Transwell™ chamber assay with Matrigel™ as a barrier.

All tested modulators stimulated differentiation in terms of increased secretion of hCG. In parallel, adhesion to endometrial cells was always reduced, whereas adhesion to ECM components was not altered. Invasiveness, in contrast, could be affected in both directions depending on the modulator used: e.g. RA and PMA increased, MTX and cAMP decreased invasion of JAR cells.

These results suggest an inverse correlation between differentiation of trophoblastic (choriocarcinoma) cells and their adhesiveness for host cells. The relation between differentiation and matrix penetration appears to be more complex, possibly depending on the pathway of signal transduction that is influenced by the used modulators of differentiation.

Supported by the Dr.-Mildred-Scheel-Stiftung für Krebsforschung.

CARBOHYDRATE MOIETIES ON THE SURFACE OF EPIDIDYMAL SPERMATOZOA OF THE CAT

Th-155 H.K. Bains^a, G. Werner^b, M.P. Bansal^a & S.R. Bawa^a.

^aDepartment of Biophysics, Panjab University Chandigarh, India. ^bMedical Biology, University of Saarland, Homburg, Saar, Germany.

During the passage of the spermatozoa in the epididymis, the sperm plasma membrane undergoes transformation. Herein we present the changes in the carbohydrate moieties on the plasma membrane of cat (*Felis catus*) spermatozoa during epididymal maturation.

Fluorescein isothiocyanate (FITC) linked lectins Concanavalin A (Con A) and Triticum vulgaris or wheat germ agglutinin (WGA) were used to label the spermatozoa. Samples were examined microscopically (*Olympus Vanox S*) and flow cytometrically (Beckton Dickinson).

With FITC-Con A there is an increase in the labeling as the spermatozoa move from the caput to cauda regions of the epididymis. Preponderance of Con A binding sites on the caudal spermatozoa suggests a possible role of these sites during fertilization. WGA binding decreases during epididymal passage and the receptors get restricted to the equatorial segment of the spermatozoa. This signifies a definite remodelling of the sperm plasma membrane during maturation of cat spermatozoa since the receptors on the equatorial segments have an important role in sperm zona recognition and binding events.

LECTIN HISTOCHEMISTRY OF THE OLFACTORY SYSTEM IN *Lepisosteus oculatus*

Th-154 V. Franceschini, M. Lazzari and F. Ciani
Department of Biology, University of Bologna, Via Belmeloro 8, I-40126 Bologna, Italy

The Holostei are generally considered a primitive group of fish of which only a small number of species have survived. This superorder of Actinopterygii was replaced in the evolutionary development by the Teleostei, the largest and most versatile group of modern ray-finned fishes.

We have studied the distribution and density of specific terminal carbohydrate moieties of the surface glycoproteins in the primary olfactory neurons and their projections in the olfactory bulbs in *Lepisosteus oculatus*, a representative species of Holostei.

Paraffin sections of olfactory epithelium and olfactory bulbs were incubated with the following HRP-conjugated lectins: *Glycine max* agglutinin (SBA), *Bandeiraea simplicifolia* agglutinin (BSA-I and its B4 isomer, BSA-I-B4), and *Dolichos biflorus* agglutinin (DBA).

The primary olfactory neurons of *Lepisosteus* are stained by SBA, BSA-I and BSA-I-B4 but not by DBA. The lectin binding patterns indicate that the cell surface of the olfactory neurons of *Lepisosteus* are characterized by a high density of α-D-galactose residues. We have previously demonstrated that in other actinopterygian species the membranes of the primary olfactory neurons and their synaptic terminals in the olfactory bulbs, called "glomeruli", possess glycoproteins with α-D-galactose and α-N-acetyl-D-galactosamine residues. This different molecular characteristic in the terminal carbohydrate moieties of cell surface glycoproteins of primary olfactory neurons between actinopterygian species defies explanation. An analogous, different behaviour in the carbohydrate moieties was demonstrated by us between urodeles and anurans and between *Ambystoma* and *Triturus*, two urodele species. The presence of these carbohydrates on primary olfactory neurons suggests that these surface glycoproteins play a role in the growth, fasciculation and orientation of the axons during olfactory receptor development and turnover.

CELLULAR EXPRESSION OF ABH BLOOD GROUP DETERMINANTS IN THE COURSE OF

Th-156 HUMAN FOETAL DEVELOPMENT

V. Sarafian^a, T. Marinova^b, H. Taskov^c,
P. Dimova^d and I. Georgiev^d

^a Department of Biology, Medical University, Plovdiv, ^bDepartment of Biology, Medical Institute, Sofia; ^cNational Centre of Infectious & Parasitic Diseases, Sofia; ^dDepartment of Histology & Embryology, Medical University, Plovdiv, Bulgaria

The ABH blood group antigens are genetically determined carbohydrates with various cellular localization and still undefined biological functions.

The expression of ABH antigens in human embryos and foetuses (6-18 weeks of gestation) was studied. The PAP, the immunogold and the immunogold-silver enhancement techniques were applied. As primary antibodies were used the monoclonals to ABH antigens produced by us.

Positive immunostaining was always noticed in mesenchyme cells even before their transformation into endothelial. Modulation in the cellular expression of ABH antigens was observed in erythroblasts and erythrocytes. The immuno-electron microscopy of foetal thymus revealed blood group determinants in areas of desmosomal contacts and in zones of close proximity between lymphocytes and epithelial cells. The possible role of blood group carbohydrates as immunomorphologic markers of the endothelial differentiation of mesenchyme cells is discussed. The participation of ABH determinants in cellular interactions is also considered.

Th-157 EXPRESSION OF SPECIFIC SURFACE OLIGO-SACCHARIDES AS EARLY MARKERS OF INTRAMEMBRANOUS OSSIFICATION

A. Zschäbitz^a, H.J. Gabius^b, H. Koepp^c, E. Stofft^a

^a Institute of Anatomy and Cell Biology, Mainz, Germany; ^b Institute of Physiological Chemistry, München, Germany; ^c St. Josef's Hospital, Wiesbaden, Germany

A complex pattern in the expression of extracellular proteoglycans and glycoproteins has long been recognized as a stage dependant element of bone development. In contrast, only few studies have been reported, analyzing distribution of oligosaccharid structures and endogenous lectins in the course of intramembranous ossification. Therefore, we have investigated the fetal development of the maxilla in rats. The binding characteristics of monoclonal antibodies, (exogenous) lectins and neoglycoproteins were determined in cryostat and paraffin sections (without/with enzymatic pretreatment). The results obtained indicate that loss of terminal sialic acid molecules is an early and specific sign of the beginning ossification preceding the process of calcification. The expression of terminal non-sulfated resp. non-acetylated β -N-acetylglucosamine also was restricted to evolving bone. The development was associated with an increase of intramembranous N-acetylglucosamine-(β 1,4)-N-acetylglucosamine. Terminal galactose-(β 1,3)-galactosamine was characteristic for bone blastea but was lost during osteogenic tissue formation. Receptors binding sialic acid resp. galactose-6-phosphate were detected in the cytoplasm of osteoblasts as well as in the extracellular matrix. No correlations were found between the time- and position-specific changes of endogenous lectins resp. specific oligosaccharides and the distribution patterns of fibronectin, bone sialoprotein, osteonectin, decorin, type I collagen and alkaline phosphatase. Taken together, these data suggest that membranous ossification is not only correlated with specific changes in the deposition of matrix components but also alterations of glycoconjugate structures.

DEVELOPMENTAL ROLE OF SURFACE GLYCOCOCONJUGATES IN EMBRYOS AND LARVAE OF AN ANURAN

G.Faraldia^a, A. Mauceri^b, C. Bassolia^a, G. Tagliaferro^a

^aInstitute of Comparative Anatomy, University, Genova, Italy; ^bDepartment of Animal Biology and Marine Ecology, University, Messina, Italy.

Rana dalmatina embryos and larvae were studied, by the use of several FITC-labeled lectins, to investigate the localization of surface oligosaccharides involved in cell adhesion and morphogenetic movements during development and their functional change.

During early developmental stages, when cell migration takes place (gastrulation, neural crest migration), both WGA- and ConA-binding-sites were found in ectodermal cells, while WGA, ConA and PNA labeled the envelopes or the perivitelline space.

During later developmental stages, from neurula to hatching, the distribution patterns of WGA-, ConA- and PNA-binding sites were substantially the same as observed in the gastrula. LPA-affinity sites begin to appear in ectodermal cell adhesion regions, together with the differentiation of a two-layer epithelium.

In the post-hatching stages, ectodermal derivatives, such as larval skin, eyes, and central nervous system, were strongly labeled with WGA, ConA and LPA; at the same time, PNA-affinity sites disappeared.

The lectin-binding glycoconjugates visualized during the precocious developmental stages are known to be involved in the regulation of cell recognition and adhesion, while the LPA-binding sites seem to be related to the establishment of the cell adhesion junctions. Thus, the different expression of glycoconjugates during development can be considered as a marker of the transient cellular behaviour, related to the different morphogenetic events.

Th-159 FUCOSYLATIONS OF TYPE 1 AND TYPE 2 GLYCOCOCONJUGATES DURING HUMAN EYE AND SKIN DEVELOPMENT

J.J. Candelier^a, R. Mollicone^a, B. Mennesson^b, I. Reguigne^a, P. Couillin^a and R. Oriol^a

^aINSERM U178, 16 Av Paul-Vaillant Couturier, 94807 Villejuif Cedex, France; ^bHôpital de Pontoise, 95300 Pontoise, France.

From 38 days on, type 1 precursor (Le^a) and $\alpha 1 \rightarrow 2$ Gal fucosylated type 2 (H type 2) were expressed in ectoderm and lens placode. The $\alpha 1 \rightarrow 3$ GlcNAc fucosylated type 2 precursor (Le^x) was observed on ectoderm but not on the placode. With differentiation (10 weeks) the patterns of Le^a and H type 2 were progressively fucosylated in $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 3$ GlcNAc to become Le^a and Le^y respectively. The Le^y epitope was not present on the lens placode. Therefore, the ectoderm, which has been induced by a complex series of interactions to form the lens placode has a particular fucosylation pattern. At 20 weeks skin and developing cornea Le^a antigen was first fucosylated in $\alpha 1 \rightarrow 2$ (H type 1) and later, in $\alpha 1 \rightarrow 4$ (Le^b) in sweat glands and cornea. Alternatively, type 2 structures persist as H and Le^y antigens. In adult tissues the Le^y antigen disappears from cornea but remains in sweat glands. At this stage all the type 1 structures were observed in sweat glands, but on the cornea only the Le^a epitope was expressed. Overall, the results suggest that terminal differentiation of human eye and skin is associated with a cascade of ordered fucosylations related to the induction processes.

Th-160 OLIGOMANNOSIDIC GLYCAN MEDIATED INTERACTION BETWEEN NEURAL CELL ADHESION MOLECULES INDUCES SIGNAL TRANSDUCTION

L.Griffith¹, P.Heiland¹, M.Schachner² and B.Schmitz¹

¹Biochemical Laboratory of the Institute of Anatomy and Physiology of Domestic Animals, University Bonn, FRG and ²Department of Neurobiology, ETH Zuerich, Switzerland

Oligomannosidic glycans N-glycosidically linked to the neural cell adhesion molecule L1 mediate association with the neural cell adhesion molecule NCAM. That this association is implicated in neurite outgrowth we could show by adding oligomannosidic glycans to the culture medium of murine early postnatal cerebellar neurons, which disturbed the L1/NCAM interaction resulting in a strongly reduced neurite outgrowth (Horstkorte et al., J. Cell Biol. 121, 1409, 1993).

Based on observations suggesting that phosphorylation of L1 is at least partially dependent on this particular interaction, signal transduction mechanisms are studied with the aim to elucidate the intracellular responses elicited by the glycan mediated L1/NCAM interaction which ultimately influence neurite outgrowth.

Th-163 NUCLEAR CYTOPLASMIC INTERACTION OF MOUSE
ENUCLEATED GV-STAGE AND SPERMATOGONIC
CELLS

M. Kubelka^a, R.M. Moor

The AFRC Babraham Institute, Babraham, Cambridge CB2 4AT, U.K.; ^bInstitute of Animal Physiology and Genetics, Dept. of Genetics, 277 21 Libechov, Czech Republic

In our study we show that the MPF activity required for PCC develops fully in cytoplasts obtained by enucleation of germinal vesicle (GV)-stage oocytes. GV-stage oocytes were obtained from 4-Bwk old CFLP a/a females stimulated with 10IU PMSG, 48hr after PMSG injection. Oocytes were cultured in Medium 199 at 37°C in 5% CO₂ environment, and the breakdown of germinal vesicle (GVBD) was prevented by addition of 200μg/ml dibutyryl cyclic AMP. In additional experiments protein synthesis was also blocked by 10μg/ml cycloheximide. Oocytes were enucleated after 1hr pretreatment with 7μg/ml cytochalasin B in dbcAMP supplemented medium using micromanipulation. The enucleated oocytes were cultured either in control medium, or in medium supplemented with dbcAMP and dbcAMP + cycloheximide, and after 16hr of culture they were checked for the presence of MPF. Ooplasts cultured in control medium developed MPF activity, as demonstrated biochemically by an increase in H1 kinase activity and biologically by their capacity to induce GVBD and chromosome condensation when fused to intact GV-stage oocytes and cultured in the presence of dbcAMP. In contrast, no such evidence of MPF activity was found in ooplasts cultured either in the presence of dbcAMP, or in the presence of dbcAMP and cycloheximide. Ooplasts obtained by enucleation of GV-stage oocytes were used for fusion with spermatogonia and pachytene spermatocytes. Ooplasts and spermatogenic cells, in 0.3M glucose medium containing 0.1mM CaCl₂ and 0.1mM MgSO₄, were exposed to 6V AC pulse for 3sec and 10C pulse of 50μsec of 1kV/cm using Zimmerman's fusion apparatus. The fusion rate obtained for ooplasts and pachytene spermatocytes was 32% (21/65), and for ooplasts and spermatogonia was 9% (3/34). After 16hr of culture all hybrids were morphologically examined. PCC was found in 71% (15/21) of pachytene-derived hybrids, in 29% (6/21) the donor cell nucleus remained intact after fusion. In spermatogonia-derived hybrids intact nuclei were found in all but one hybrid in which uneven distribution of condensed chromosomes was seen. The results suggest the MPF activity developing in ooplasts interacts with donor nucleus, resulting in PCC at least in pachytene spermatocytes nuclei.

Th-165 KINETICS OF ENTRY INTO DNA SYNTHESIS
AND CORRELATION OF SIBLING GI TRANSIT
TIME OF MAMMALIAN PK CELLS IN
MONOLAYER CULTURE

V. Sekharov, L. Voronkova

Institute of Chemical Physics, Russian Academy of Sciences, Kosygin Str. 4, Moscow 117977, Russia

The proliferation kinetics of cultured pig embryo kidney cells were studied by time-lapse microphotography and [³H]TdR autoradiography. The duration and variability of all phases of the cell cycle were estimated. Evidence is presented that variation in cell cycle transit time of both unrelated and sibling cells results mainly from variation in transit of GI phase. The results indicate that the course of the entry into S phase is compatible with the first order kinetics and with the "transition probability model" of cell cycle control with location of the random transition in GI. In accordance with random occurrence of an event the sister cells showed frequently (up to 46%) the difference in isotope labeling. Nevertheless, the analysis of labeling pattern of sister cells indicates the clear correlation of sister cells GI transit time. This correlation may be result of interaction between sister cells. The idea of such interaction was supported by the results of our experiments with UV microirradiation of single cells. It has been demonstrated that UV-induced delay in cell cycle of irradiated sister cell led to delay in cell cycle of unirradiated sister cell as well.

Th-164 INDUCTION OF CELL CYCLE EVENTS
BY GROWTH FACTORS AND PROTEIN
SYNTHESIS INHIBITORS

I.Rosenwald^a, N. Setkov ^b and
O. Epifanova ^c

^a Division of Health Sciences, Harvard-MIT, Cambridge, USA; ^b Institute of Biophysics, RAS, Krasnoyarsk, Russia; ^c Institute of Molecular Biology, RAS, Moscow, Russia

It has been shown by Northern blot analysis that brief exposures of resting (serum-deprived) NIH 3T3 cells to either cycloheximide (10 μg/ml) or puromycin (20 μg/ml) induce a transient expression of c-fos, c-jun and c-myc in a way similar to that following stimulation with PDGF (50 ng/ml). Concomitant addition of actinomycin D to the cultures abrogated the induction of protooncogenes.

Cell fusion studies revealed that pretreatment of resting cells with either PDGF or protein synthesis inhibitors abolishes their growth suppressive capacity towards proliferating (serum-stimulated) cells in heterokaryons. However, the observed effects of PDGF disappeared in the presence of actinomycin D, whereas the effects of cycloheximide and puromycin did not.

The results suggest that in the course of mitogenic stimulation extracellular growth factors and protein synthesis inhibitors may participate in the same or in different but overlapping intracellular pathways.

Th-166 DIFFERENTIATION OF AVIAN RED CELLS
- HD3 CHL LINE AS A MODEL SYSTEM

M. Grdiša¹ and D. Bešlo²

¹Department of Molecular Medicine, Ruder Bošković Institute, Zagreb, Croatia

²Faculty of Food and Technology, Osijek, Croatia

Development of the erythroid cells is accompanied by changes in the composition and properties of the plasma membranes of these cells. HD3 cells are chicken erythroblast transformed with temperature sensitive erythroleukemic virus. In the course of HD3 cells differentiation, the transports of nucleoside and sugar were investigated. The enzyme responsible for metabolism of sugars and nucleosides were studying. Cells differentiate into a red cells upon temperature shift to 42°C in the presence of inducers. The level of nucleoside transporter increases, before elevated level of Hb are detected. Glucose transport activity falls upon induction of HD3 cells. The activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glucose 6-phosphate dehydrogenase (GPDH) changes upon induction. Using cDNA probe for chicken GAPDH, decreasing of mRNA was seen after induction of differentiation. The changes in glucose and nucleoside transport levels show that nucleosides provide a major source of oxidisable carbon compounds to sustain metabolism in mature chicken red cells.

GLYCOCOCONJUGATE CHANGES IN AN EMBRYONIC HUMAN EPITHELIAL LINE DURING THE CELL CYCLE

Th-167

A.M. Bolognani Fantin^a, A. Franchinio^a, A.M. Fuhrman Contib, R. Malgarab^b and B. Rebecchia^b^aDipartimento di Biologia Animale, Università di Modena (Italy); ^bDipartimento di Biologia e Genetica per le Scienze Mediche, Università di Milano (Italy)

Cell surface and cytoplasmic glycoconjugates were studied in embryonic human explant (EUE) cells (a transformed heteroploid line) synchronized in the cell cycle phases in order to study the changes in the expression and localization of these compounds. As previously demonstrated (Bolognani Fantin et al., 1989) a well developed cell coat is present over the plasma membrane surface of EUE cells, and a noticeable glycoconjugate component is also present in the cytoplasm. Cytochemical stainings with specific lectins and immunocytochemical reactions selective for several glycoconjugates and adhesion molecules we carried out. The following results were obtained: 1) the affinity for all the tested lectins both on plasma membrane and cytoplasm was stronger in G1 and G2 phases than in the S one; 2) a rearrangement of cytoplasmic glycoconjugates was observed in the S phase as lectin affinity was frequently restricted to an area around the nucleus; 3) the nucleus was stained particularly with WGA in G1 and G2; 4) cells in mitosis were always strongly stained by lectins with exception of PNA; 5) the immunocytochemical reactions were positive for heparan sulfate (with strong reaction in S and G2) and cellular fibronectin, poorly positive for dermatan sulfate proteoglycan and L-CAM.

Bolognani Fantin et al., *Histochem. J.*, 21, 79-88, 1989

SOME CHARACTERISTIC FEATURES OF THE G2-PHASE MITOSIS ARREST OF MOUSE EMBRYOS IN VITRO. NEW MODELS FOR THE '2-CELL BLOCK' RESCUE.

Th-169 G.G. Sekirina, I.E. Neganova, N.A. Bogoliubova
Laboratory of Cell Morphology, RAS,
Institute of Cytology, St.Petersburg, Russia

During second cell cycle as in vivo as in vitro blocking genotypes (BALB/C) embryos have similar dynamics of the increase of pronucleoli number and of the content of Ag-proteins. In vivo G2/M phase of these embryos show the concentration of Ag-proteins on the nucleus contour. At the same time in vitro this picture can not be seen, but some light clusterization and accumulation of mitochondria near plasma membrane are observed.

Two or three days later, when non-blocking genotypes (CBAK C57BL/F1) embryos reach to blastocyst stage, capacity to accept vital dye (Rd - 123), but they have a state of deep block with rough mitochondria clusterization and strong cluster redistribution to periphery of blastomeres.

It is known that inbred strain mouse embryos have in vitro so-called '2-cell block' on the G2-phase of second cell cycle after inclusion of embryonic genome. Described above features of this block indicate that it is not prolonged G2-phase, but peculiar progressing state of blocking genotype embryos in vitro, which cell cycle in vivo can last several days.

Three ways were used to rescue of block: 1. culture of blocking embryos in medium conditioned by non-blocking embryos, 2. co-culture blocking and non-blocking embryos, 3. 2 cell-->2 cell and 2 cell-->8 cell aggregation chimeras between blocking and non-blocking embryos. Blocking embryos were placed into rescue conditions at the G1-phase of second cell cycle, i.e. before first burst of embryonic genome expression and many hours earlier than the block starts.

Highest (up to 100%) and almost complete rescue effect with development of blocking embryos in vitro to 8-cell or blastocyst stage (in accordance duration of cultivation) was obtained in aggregation chimeras.

Comparative analysis of results received in these three experimental models suggest that some embryonic origin factors participate in this block rescue, but adhesive cell-cell interactions have a leading role in aggregation chimeras to carry out this effect.

PROGRESS OF DIVISIONAL MORPHOGENESIS OF A CILIATE

TETRAHYMENA PYRIFORMIS IS CONTROLLED BY THREE

PHOSPHORYLATION-DEPENDENT SWITCH POINTS

J.Kaczanowska^a, L.Buzarista^a, W.Krawczyńska^band D. Wheatley^c^aDepartment of Cytophysiology, Warsaw Univ., Warsaw 00-927, Poland;^bNencki Institute of Exp. Biol., Warsaw 02-093, Pasteur 3, Poland;^cDepartment of Pathology, Univ. Aberdeen AB2 2D9, U.K.

During interphase of *Tetrahymena*, the oral apparatus (OA) showed the phosphorylated oral center detected with the MPM2 antibody against phosphorylated epitopes of mitotic cells (Davis and Rao, 1987) associated to the postoral microtubules and to the area engaged into phagocytosis. During cell division, the parental OA transiently regressed. During and after cytokinesis two OAs for offsprings showed the phosphorylation of the oral centers, produced the postoral microtubules and started to feed. The inhibitor of protein kinases, 6-dimethylaminopurine (6-DMAP) suppressed the mitosis-specific phosphorylations of a number of polypeptides found in the triton-soluble cytoskeletal frameworks of *Tetrahymena pyriformis* with only slight impact on protein synthesis.

6-DMAP differently acted on *Tetrahymena* depending upon the advancement of cells in cell division at the moment of introducing to 6-DMAP:

1) applied to early dividers induced cells to switch from cell division to an abortive oral replacement pathway resulting in the undivided cells with defective OAs. These abnormal OAs were deprived of the oral center, unable to nucleate the postoral microtubules and unable to feed.

2) applied to cells committed to divide, brought about the arrest of cells in cytokinesis with two OAs deprived of the phosphorylated oral centers without the postoral microtubules and unable to feed.

3) in early furrowing cells, 6-DMAP did not affect cleavage, but inhibited the phosphorylation of the oral centers, the nucleation of postoral microtubules the start of phagocytosis and stabilization of the OAs.

1) Davis F.M. & Rao P.M. 1987 Antibodies to mitosis-specific phosphoproteins. In: *Molecular Regulation of Nuclear Events in Mitosis and Meiosis*, eds. R.A. Schlegel, K.S. Halleck and P.M. Rao. Acad. Press, Orlando pp. 259-293.

THAPSIGARGIN ARRESTS THE CELL CYCLE IN Th-170 EARLY SEA URCHIN EMBRYOS.

M.G. Wilding and M.J. Whitaker

Department of Physiology, University College London, Gower Street, LONDON WC1E 6BT, U.K.

We are looking at the role of intracellular calcium ions in cell cycle regulation in embryos of the sea urchin *Lytachinus pictus*. Previous data suggest that calcium released from intracellular stores may regulate entry into mitosis (Hafner and Petzelt, 1987; Whitaker and Patel, 1990 for review). We have used thapsigargin, an inhibitor of the calcium-ATPase of the endoplasmic reticulum, in order to deplete the intracellular stores of calcium and to demonstrate the involvement of calcium in cell cycle regulation. We find that microinjection of 60μM thapsigargin will deplete the IP₃ and calcium ionophore (4-Br A23187)-releasable calcium stores and it also activates a calcium influx pathway. Equivalent concentrations of another calcium-ATPase inhibitor, cyclopiazonic acid, gave similar results. 60μM thapsigargin also blocks the cell cycle at entry into mitosis. Microinjection of 10μM thapsigargin only partially depletes this store and does not block the cell cycle. These data suggest that the thapsigargin-sensitive calcium store is involved in cell cycle signalling.

Whitaker, M. and Patel, R. (1990). Calcium and cell cycle control. *Development* 108: 525-542.

Hafner, M. and Petzelt, C. (1987). Inhibition of mitosis by an antibody to the mitotic calcium transport system. *Nature* 330: 264-266.

Th-171 IS CYTOKINESIS IN *Saccharomyces cerevisiae* REGULATED BY A PROTEIN KINASE CASCADE?
Fatima Cvrčková and Kim Nasmyth

I.M.P., Dr. Bohr-Gasse 7, A-1030 Wien, Austria

The simultaneous onset of DNA replication, spindle pole body duplication and budding at the end of G1 in the yeast cell cycle (known as Start) requires the Cdc28 protein kinase associated with at least one of three G1 cyclins (Cln1 to Cln3). These cyclins are "functionally redundant" but not equal; e.g. Cln1 and Cln2 are more important for budding than for DNA replication (Cvrčková and Nasmyth, EMBO J. 12:5277, 1993).

Mutations in *CLA4* and *CLA10* lead to similar lethal cytokinetic defects in cells lacking Cln1 and Cln2 but not in wild-type cells, suggesting that Cln1 and/or Cln2 have a direct role in preparation for cytokinesis. *CLA10* is allelic to *CDC12*, a gene encoding a component of the microfilament ring which assembles around Start at the site of bud emergence and is subsequently needed for cytokinesis. Both *cla4* and *cla10* mutants may be defective in forming this ring.

CLA4 codes for a protein kinase similar to the mammalian Pak kinase (which interacts with a homologue of a yeast protein required for budding - see Manser et al., Nature 367:40, 1994) and to the yeast Ste20 kinase. Ste20 is required for transduction of the pheromone response signal from a receptor to a kinase cascade analogous to the mammalian MAPK system. We have found that Ste20 is essential in cells lacking *CLA4*, indicating that Ste20 can compensate for the loss of *CLA4* function and suggesting that targets of *CLA4* may be similar to those of Ste20.

Although *CLA4* is not essential, *cla4* mutants suffer from a morphogenetic defect. This defect disappears in high salt media which induce another MAPK-like pathway - the HOG (high osmolarity glycerol) system; the rescue by high salt requires an intact HOG pathway. This observation provides further support for the hypothesis that *CLA4* regulates an unknown MAPK-like kinase cascade.

Th-173 POLYPHOSPHOINOSITIDE METABOLISM DURING MITOTIC DIVISIONS OF SEA URCHIN EGG

B. Ciapu* and D. Pesando**

* Laboratoire de Physiologie Cellulaire et Comparée, Faculté des Sciences, Parc Valrose, BP 71, 06108 Nice Cedex 02; ** Unité INSERM 303, BP 3, 06230 Villefranche-sur Mer FRANCE

Changes in intracellular free calcium (Ca²⁺) have been shown to punctuate the cell cycle in various dividing cells including mammalian or plant cells, and eggs from mouse, *Xenopus* and sea urchin. These Ca²⁺ transients can be detected after cellular activation and at the time of pronuclei migration, nuclear envelope breakdown (NEB), metaphase-anaphase transition and cytokinesis. We have investigated whether this event could be related to variations in polyphosphoinositide metabolism.

Eggs were fertilized in the presence of [³²P]-orthophosphate and the incorporation of this precursor into phosphatidylinositol phosphate (PIP) and phosphatidyl-inositol bisphosphate (PIP₂) was measured. While [³²P]-orthophosphate accumulation into eggs accelerated with time, its incorporation in inositol-containing lipids fluctuated during the first four cell cycles. Peaks of incorporation of the precursor into PIP₂ occurring during the first cell cycle corresponded to morphologic events (pronuclear migration, streak stage and cleavage) and to transient increases of intracellular free calcium reported by others (Whitaker and Patel, 1990, Development 108, 525-542). The following peaks corresponded to the second, third and fourth cleavages. We observed peaks of incorporation of ³²P into PIP before the second, third and fourth cleavages. These oscillations in PPI metabolism correspond to repetitive increases in intracellular amount of IP₃.

Fluctuations in PPI and inositol phosphates still occurred in eggs incubated in the presence of inhibitors of protein synthesis, emetine and nordidemnin, which arrest mitotic divisions during the first and the second cell cycle respectively. We conclude that fertilization induces PPI metabolism to oscillate independently of protein synthesis, the intensity of the oscillations being however dependent on protein synthesis. Our observations point to the PPI metabolism as an important component of the cell cycle regulation in sea urchin eggs.

Th-172 DNA SYNTHESIS IN LYMPHOCYTES EXPOSED TO MERCENENE, A DRUG OF MARINE ORIGIN

A.C. Schmeer^a, V. Mareš^{b,d}, H. Kozáková^c

, D. Horton^e, and V. Lisá^b

^aMercenene Cancer Research Inst., New Haven, CT, USA;
^bInst. of Physiology, Czech Acad. Sci., Prague, CR; ^cInst. of Microbiology, Czech Acad. Sci., Prague, CR; ^dDept. of Zoology, Kuwait University, Kuwait City, Kuwait and ^eDept. of Chemistry, American University, Washington, DC, USA.

Mercenene is a hydrophilic extract from *Mercenaria* m. (marine clam) which inhibits DNA synthesis and the growth of some tumors *in vivo* and *in vitro*. We report here the drug's dual effect on DNA synthesis in rat and mouse spleen lymphocytes in short term cultures.

The lymphocytes were isolated from the young adult rats and mice and cultured in microwell plates in serum-free medium, with or without Concanavalin A (Con A) or *E. coli* lipopolysaccharide (LPL). Mercenene was administered at several concentrations (50 to 500 µg/ml) at the time of seeding the cells. DNA synthesis was assessed by ³H-thymidine incorporation measured by liquid scintillation counter in 48 h cultures. It was found that low doses of Mercenene (50 and 100 µg/ml) profoundly stimulated DNA synthesis in cells cultured without being pre-activated with Con A or LPL. Mercenene caused some stimulation of DNA synthesis at a much lower degree in cultures pre-activated by Con A or LPL. A higher dose of Mercenene (500 µg/ml) led to inhibition of DNA synthesis in all types of cultures.

The data suggest that Mercenene effects DNA synthesis in both T- and B-lymphocytes. Stimulated DNA synthesis following treatment with low doses of Mercenene may represent a new mechanism of activity for Mercenene which caused regression and inhibition of experimental tumors *in situ* observed and reported earlier.

Th-174 SEPARATION OF MULTIPLE GENES CONTROLLING THE T CELL PROLIFERATIVE RESPONSE TO IL-2 AND ANTI-CD3 INTO DIFFERENT RECOMBINANT CONGENIC STRAINS

M. Lipoldová⁺, A. Zajíčková⁺, V. Holán⁺,
A.A.M. Hart^X, M. Kosařová⁺, M. Krulová⁺, and P. Dámant^X

⁺Inst. Molec. Genetics, Prague, Czech Republic, ^XThe Netherlands Cancer Institute, Amsterdam, The Netherlands

The lymphocytes of the strain BALB/cHeA exhibit a low proliferative response to IL-2 and a high response to anti-CD3 mAb, while the strain STS/A lymphocytes response to these stimuli is the opposite. We have analyzed the genetic basis of this interstrain difference using a novel genetic tool: Recombinant Congenic Strains (RCS). Twenty BALB/c-c-STS/Dem (CcS/Dem) RCS were used, each containing a different random set of approximately 12.5% of the genes from STS and the remainder from BALB/c. Consequently, the individual genes participating in the multigenic control of a phenotypic difference between BALB/c and STS become separated into different CcS strains where they can be individually studied. The strain distribution patterns of the proliferative responses to IL-2 and anti-CD3 in the CcS strains are different, showing that different genes are involved. The large differences between individual CcS strains in response to IL-2 or anti-CD3 mAb indicate that both reactions are controlled by a limited number of genes with a major effect. The high proliferative response to IL-2 is a dominant characteristic intrinsic to lymphoid cells, rather than being caused by increased cell numbers of a particular cell subset. The response to anti-CD3 is known to be controlled by polymorphism in Fc_γ receptor 2 (Fcgr2) and the RCS carrying the low responder Fcgr2 allele indeed proliferated weakly. However, additional genes also strongly influence this response.

DIFFERENCES BETWEEN NEWBORN AND ADULT MICE IN THEIR RESPONSE TO MITOGENS AND INTERLEUKIN-2

Th-175 M. Lipoldová, A. Zajícová, M. Kosařová and V. Holán

Inst. of Mol. Genetics, Acad. of Sciences of the Czech Rep., Flemingovo nám. 2, 165 37 Praha 6, Czech Rep.

The immune system of newborn animals has been shown to be non-reactive or profoundly hyporeactive in a wide array of *in vitro* and *in vivo* immunological tests. Both the immaturity of the components of immune system and the presence of inhibitory cells and/or molecules in newborns have been considered as a reason for this hyporeactivity of newborns.

Newborn animals are more susceptible to fungal, viral and bacterial infections than adults, but they nevertheless survive in conventional conditions. One of the mechanisms ensuring their survival could be the synthesis of cytokines with inflammatory and cytotoxic functions. Newborn spleen cells synthesize considerable amounts of interleukin(IL)-1 and tumour necrosis factor (TNF)- α mRNA after lipopolysaccharide (LPS) stimulation.

In this paper we have extended our studies to induction of molecules with immunoregulatory functions in splenocytes by mitogens Con A and LPS and by growth factor IL-2. We have found that LPS and IL-2 have ability to induce synthesis of IFN- γ and IL-10 mRNA in spleen cells both from newborn and adult mice. Con A failed to induce TNF β and IFN- γ in splenocytes newborn mice, but was able to induce synthesis of a high level of IL-10 mRNA in these cells. Since IL-10 is a cytokine with inhibitory functions, presence of IL-10 and simultaneously absence of some other immunoregulatory molecules could be responsible for phenomena observed in cultures of neonatal spleen cells.

INTRACELLULAR POSITION OF ProTa IN CELLS
GOING THROUGH THE CELL CYCLE

Th-177 K. Vareli, O. Tsolas, M. Frangou-Lazaridis

Laboratory of Biological Chemistry, University of Ioannina Medical School, Ioannina GR 451 10, Greece

Prothymosin α (ProTa) is a small and extremely acidic nuclear protein of 109 to 111 aminoacids. It has broad species and tissue distribution and it is indispensable for mitosis to proceed, as it was shown by antisense oligonucleotides. ProTa has been found covalently linked to small cytoplasmic RNA but was also isolated as an HMG-protein. Aminoacid sequence analysis indicated a nuclear localization signal at the carboxyterminus of the protein which was also found to be transcriptionally activated by the c-myc oncogene in functional studies.

We present evidence on ProTa mRNA and protein levels during the cell cycle. We found a relationship between ProTa expression and DNA synthesis as well as a correlation with c-myc and cyclins A and B expression. The intracellular position of the protein during the cell cycle was also followed with immunofluorescence.

ROLES FOR ECDYSTEROIDS AND GROWTH FACTORS IN THE CONTROL OF THE CYCLE OF INSECT EPIDERMAL CELLS

Th-176 P. J. Hatt^a, M. Morinière^a, H. Oberlander^b and P. Porcheron^a

^aLaboratoire de Biochimie, URA CNRS 686, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France; ^bInsect Attractants Laboratory, ARS, USDA, PO Box 14565, Gainesville, FL 32604, USA

During post-embryonic development of insects, molting cycles affect epidermal cells with alternate periods of proliferation and differentiation. Cells of the IAL-PID2 cell line, established from imaginal discs of a moth, when exposed to the molting hormone, 20-hydroxyecdysone, accumulated in the G2 phase of their cycle, formed epithelial-like aggregates and synthesized specific proteoglycans. These results are meaningful in terms of control of insect epidermal differentiation by ecdysteroids. However, the cytostatic effect of 20-hydroxyecdysone was shown to be reversible, and, prior exposure of the cells to the hormone allowed the cells to accomplish afterwards at least two cell cycles in serum-free medium. This suggests that in this case production of autocrine growth factors induced by the hormone could circumvent the absence of serum. Recent discovery of members of the insulin superfamily in insects led us to check the potential effects of IGFs on IAL-PID2 cell cycle regulation. Results obtained showed that IGFs indeed induced resumption of the cell cycle after an arrest in G1 caused by serum deprivation. Therefore, this cell culture model provides potential for further studies of the interactions between ecdysteroids and growth factor homologs in insect epidermal cells during molting cycles.

4-PYRANONE DERIVATIVES: A NEW CLASS

Th-178 OF COMPOUNDS WITH ANTILEUKEMIC ACTIVITY

J. Bransová^a, J. Brtko^a, M. Uher^b, and L. Novotný^c

^aInstitute of Experimental Endocrinology, Slovak Academy of Sciences, 833 06 Bratislava, ^bDepartment of Organic Chemistry, Faculty of Chemical Technology, Slovak Technical University, 812 037 Bratislava, ^cCancer Research Institute, Slovak Academy of Sciences, 812 32 Bratislava, Slovakia

A significant inhibitory effect of eight halogen derivatives of 5-hydroxy-2-hydroxymethyl-4-pyranone on L1210 murine leukemia cell growth was found when compared with the 5-hydroxy-2-hydroxymethyl-4-pyranone or its five alkyl or acyl derivatives, four nitrogen derivatives or five oxidation products. LD₅₀ was extrapolated from the growth inhibition curves at a compound concentrations ranging from 0.1 to 100 μ mol/l.

In conclusion, all halogen derivatives of 5-hydroxy-2-hydroxymethyl-4-pyranone tested inhibited the L1210 cell growth in the order: 5-hydroxy-2-iodomethyl-4-pyranone > 6-bromo-2-bromomethyl-5-hydroxy-4-pyranone > 6-bromo-5-hydroxy-2-hydroxymethyl-4-pyranone > 2-bromomethyl-5-hydroxy-4-pyranone > 5-benzoyloxy-2-chloromethyl-4-pyranone > 6-bromo-2-chloromethyl-4-pyranone > 6-chloro-2-chloromethyl-5-hydroxy-4-pyranone > 2-chloromethyl-5-hydroxy-4-pyranone when compared to 5-hydroxy-2-hydroxymethyl-4-pyranone which has no effect on L1210 cell growth.

Th - 179 CALMODULIN REGULATES PCNA EXPRESSION IN NRK CELLS ACTIVATED TO PROLIFERATE FROM G0
Antonia López-Girona, Marta Bosch, Oriol Bachs and Neus Agell
Dept. de Biología Celular, Facultat de Medicina, Universitat de Barcelona, 08036-Barcelona, Spain

The mRNAs of most proteins involved in DNA synthesis show an S phase correlated expression when mammalian cells are stimulated to proliferate from G0. This is the case of Proliferating Cell Nuclear Antigen, a cofactor of DNA polymerase δ that is essential for the synthesis of the leading and the lagging strands of DNA. Normal rat kidney cells reentering the cell cycle from quiescence start DNA synthesis at 12 h and reach a maximum at 20 h. The expression of Proliferating Cell Nuclear Antigen parallels the synthesis of DNA. Progression through the S phase was inhibited by addition of the anti-calmodulin drug W13 to the cultures 4 h after activation. W13 addition also inhibited the increase in both Proliferating Cell Nuclear Antigen protein and mRNA indicating that calmodulin regulates its expression. Using TK-ts13 cells transfected with a plasmid containing the thymidine kinase gene under the control of the human 2.8 Kb Proliferating Cell Nuclear Antigen promoter we demonstrated that this promoter is not regulated by calmodulin. The half-life of Proliferating Cell Nuclear Antigen mRNA during G1/S transition was not modified by the W13 treatment, indicating that the decrease in this mRNA found when calmodulin was inhibited is not due to a decrease in its stability. Run-on assays revealed that W13-treated cells produced predominantly short, incomplete Proliferating Cell Nuclear Antigen transcripts during S phase, while long transcripts were generated in control cells. These results indicate that calmodulin is involved in regulating the release of a transcriptional block during G1 that is partly responsible for the increased amounts of Proliferating cell nuclear antigen found during S phase.

Th - 181 CYCLIN A IS LOCATED IN RAT LIVER MICRO-SOMES DURING LIVER DEGENERATION

A.Castro, M.Jaumot, M.Vergés, N.Agell, O.Bachs and C.Enrich

Dept. of Cell Biology, Faculty of Medicine, University of Barcelona, Spain.

Cyclin A was initially characterized as a mitotic cyclin believed to function exclusively at the G₂-to-M phase transition, although several studies have recently demonstrated a nuclear localization of this protein in S phase. Moreover, despite several investigations have described cyclin A expression during G₁, no information exists about its localization and function during this early phase of the cell cycle. We have studied *in vivo* cyclin A expression and localization during rat liver regeneration after partial hepatectomy (PH). Western blot analysis revealed that cyclin A and cdk2 were induced by a PH showing maximal levels at 18 hours after surgery. Subcellular fractionation localized cyclin A and cdk2, but not cdc2 at the microsomal fraction during late G₁. To confirm these results we purified cyclin-cdk complexes from microsomal fractions obtained from control and regenerating liver using p13-Sepharose. The samples were assayed for H1 kinase activity and tested by western blot analysis with specific antibodies. The results demonstrated a marked increase of H1 kinase activity in regenerating microsomes which correlates with the presence of cyclin A and cdk2 in these fractions. We also demonstrated that cyclin A was tightly associated with microsomal membranes since it was only removed by Triton X-110 but not by NaCl or NaOH. These results suggest a putative role of cyclin A during late G₁ which would be related with microsomal function.

Th - 180 CALMODULIN AND CALMODULIN-BINDING PROTEINS IN REPLITASE COMPLEXES
M. Jaumot, N. Agell, and Oriol Bachs
Departament de Biología Celular, Facultat de Medicina, Universitat de Barcelona, Spain

Calmodulin (CaM) has been shown to be involved in the triggering of DNA replication. Not much is known about how CaM regulates this process although it has recently reported that CaM modulates the activity of DNA polymerase- α and the expression of the Proliferating Cell Nuclear Antigen (PCNA), a co-factor of DNA polymerase- δ . The concentration of CaM is increased during the mid G1 and at G1/S it translocates into the nucleus where associates to the nuclear matrix. Since the nuclear matrix is the structure where DNA replication occurs, the association of CaM with the matrix during G1/S has been related to the triggering of DNA synthesis. It has also been shown that anti-CaM antibodies added to permeabilized cells block DNA replication. To analyse whether CaM is located in the replication sites which are associated to the nuclear matrix (replisome complexes) we have purified these structures and determined the presence of CaM, CaM-binding proteins (CaMBPs), cyclins and cyclin-dependent protein kinases in these complexes. Results revealed that CaM and two CaMBPs of Mr around 130 kDa were found in the replisome complexes of human HeLa cells. Similar results were obtained in NRK cells although in these cells only a CaMPB of 140 kDa was found. These CaMBPs still remain to be identified but these results suggest that the different CaMBPs in the replisome complexes of cancer cells could be important for the expression of the neoplastic phenotype. The co-localization of CaM/CaMBPs and cyclin A/cdk2 in the replisome complexes suggest a functional relationship between CaM and the cell cycle regulators in the process of DNA replication.

Th - 182 PROTEIN KINASE C REGULATES CALMODULIN EXPRESSION IN NRK CELLS ACTIVATED TO PROLIFERATE FROM QUIESCENCE
Marta Bosch, Antonia López-Girona, Oriol Bachs and Neus Agell

Departament de Biología Celular, Facultat de Medicina, Universitat de Barcelona, Spain

When NRK cells were activated to proliferate from quiescence they started to replicate DNA 15 h after serum addition, reaching a maximum at 20 h. The highest number of cells in mitosis was observed 24 h after stimulation. We investigated here the levels of calmodulin protein and calmodulin mRNA species during proliferative activation. In homogenates from quiescent cells the concentration of calmodulin was 1.5 μ g/mg protein. At 10 h after serum addition calmodulin started to increase reaching maximum values of 3 μ g/mg protein during S phase and mitosis. Three species of calmodulin transcripts were found in quiescent NRK cells: that of 1.7 Kb from CaM I, the 1.4 Kb mRNA from CaM II and the 4.0 Kb transcript from CaM III. At 5 h after activation, the amount of all the three transcripts started to increase reaching a maximum around 20 h, being at this time 5-10 fold higher than in serum starved cells. Run-on experiments showed that in 20 h-activated cells the transcription rates of the three calmodulin genes were higher than in quiescent cells. The molecular mechanisms involved in the increase of the transcription of the calmodulin genes were analyzed. Inhibition of PKC 5 h after activation blocked the increase of the calmodulin transcripts during S phase, while inhibition of PKA did not have any effect. Addition of TPA at concentrations that did not induce DNA synthesis increased the levels of all three CaM transcripts. These evidence indicate that PKC regulates calmodulin expression when NRK cells are activated to proliferate.

Th-183 Protein kinase C is involved in mouse egg transition from the meiotic to the mitotic cell cycle
C. Tatone, R. Iorio, A. Francione and R. Colonna

Dipartimento di Scienze e Tecnologie Biomediche e di Biometria
University of L'Aquila, 67100 L'Aquila, Italy.

Activation of mouse eggs arrested at metaphase of the second meiotic division (MII) is a complex process during which the activity of maturing promoting factor (MPF) drops rapidly and the oocyte enters interphase of the first mitotic cell cycle. The transition from meiotic to mitotic control of the cell cycle can be triggered by a calcium signal and seems to require protein kinase C activation.

To investigate the actual role played by protein kinase C in mouse egg activation, we determine egg activation frequency and MPF activity in eggs challenged with the specific protein kinase C activator OAG (1-oleyl-2-acetyl-sn-glycerol) under experimental conditions allowing or preventing cytosolic calcium increase. Results provide evidence that in mouse eggs when protein kinase C is stimulated MPF inactivation and entrance into interphase do not require a calcium increase at least during the first 40 min after OAG-treatment. However, the majority of these parthenogenotes fails to exit from interphase and remains arrested at G2-phase of the first mitotic cell cycle.

The data presented here strongly suggest that in mouse eggs: 1) protein kinase C activity is involved in MPF inactivation and in the exit from M-phase; 2) the early calcium rise which occurs at the time of activation is required for the subsequent mitotic cell division.

Th-185 CELL CYCLE REGULATION OF SPECIFIC RNA-PROTEIN INTERACTIONS
M.P. Rounseville, R. Shukla and A. Kumar. Biochemistry and Molecular Biology, George Washington University, Washington, D.C.

Role of specific RNA-protein interactions in transcriptional activation and post-transcriptional regulation, such as nuclear RNA transport was studied utilizing HIV-1 regulatory genes, Tat and Rev, as experimental models. Both of these genes are unique in that they require specific RNA structural domains as *cis* acting targets. In addition, we have shown that both *Tat* and *Rev* function require human host cell cofactors which specifically interact with their RNA target domains and modulate their function. Utilizing site directed mutagenesis of the RNA target sequences we determined the sequences required for specific protein binding and, biochemically purified and isolated cDNA clones. These represent unique human genes with consensus RNA binding domains and their homologies to transcription factors.

Biological regulation of these genes with respect to cell cycle progression was studied since transcriptionally latent state of the virus is known to be induced in target cells undergoing cell growth and clonal proliferation. Primary human T-lymphocytes in G₀ phase upon mitogenic induction show sequential activation of G₁ specific cyclin-dependent kinases, as judged by their ability to induce phosphorylation of Rb gene product; and their G₁ progression is blocked by TGF β . We have shown that functional interaction of the host cell RNA binding proteins is dependent upon CDK-2/cyclin G activity and is blocked by TGF β . These results support the argument that transcriptional activation of latent viruses, require mitogenic activation of the host cells and the mechanism involves specific host-cell cofactors whose functional activation is dependent upon cell cycle regulatory kinases. These studies will enable us to focus on unique features of cell cycle modulated RNA-protein interactions in the regulation of gene expression.

Th-184 STIMULATION OF THE FIRST MEIOTIC DIVISION OF OVARIAN OOCYTES BY LOW DOSES OF RADIATION
P. Jacquet¹, L. de Saint-Georges¹, J. Vankerkom² and L. Baugnet-Mahieu¹

¹Lab. Radiobiology, Dept. Radioprotection, CEN/SCK; ²Div. Envir. Res., VITO-Mol, Belgium.

Female guinea-pigs were irradiated on days 8-10 of the 17 day-cycle, with doses of x-rays ranging from 0.25 to 4 Gy. Preovulatory oocytes were punctured from the ovaries 18 hours after irradiation. They were cultured *in vitro* for 6 hours, in order to induce their maturation and analyze their MI chromosomes for the presence of structural aberrations. When they were fixed at the end of the culture period, all oocytes irradiated with 4, 2 or 1 Gy revealed to be already in MII, and 60% of those given 0.5 Gy had also reached this stage. For oocytes irradiated with 0.25 Gy, the difference against controls was not significant (31% vs. 25%). Oocytes given 4 Gy were cultured for decreasing times, starting at 18 hours after irradiation. Collection and culture of the oocytes occurred in the presence of colchicin. Again, all fixed oocytes revealed to be at MII, whatever the duration of the culture. Furthermore, this was also true for oocytes fixed 18 hours after irradiation, without preliminary culture. Clearly, the oocytes had been stimulated *in vivo*, before removal of the ovaries. Oocytes irradiated with 2 and 1 Gy were also fixed 18 hours after irradiation, without being cultured: 71% and 63% of them, respectively, were at MII at that time. Finally, oocytes irradiated with 4 Gy were collected and cultured immediately after irradiation, for different times. The proportion of MII oocytes appeared not yet increased, 6 hours after irradiation. These results indicate that irradiation is able to induce a very rapid stimulation of the first meiotic division in maturing oocytes. The extent as well as the rapidity of such effect is dose-dependent, with an apparent threshold of 0.25-0.50 Gy (Supported by contract F13P-CT920005 from CEC).

Th-186 THE NORMAL G₂ PHASE HISTONE VARIANT SYNTHESIS PATTERN IN HEp-2 CELLS AND CHLORAMBUCIL-INDUCED CHANGES IN THIS PATTERN.

T.G. Sourlingas and K.E. Sekeri-Pataryas

NRC "Demokritos", Institute of Biology, Athens, Greece.

Histone variant synthesis has been previously analyzed and reported for many cell lines. Emphasis has mostly been given to S phase histone synthesis which is the major portion of the cell's synthesis of these proteins. The basal histone synthetic pattern of G₁ cycling cells has also been thoroughly investigated during the G₁ phase of many cell lines. G₂ phase histone variant synthesis has been given relatively little attention. Here we report the analysis of the histone variant synthesis pattern during the G₂ phase of the HEp-2 cancer cell line and the changes induced by the bisalkylating agent, chlorambucil, in the synthesis rates of the H2A and H3 histone variants.

Analysis of radiolabeled histone variants after electrophoretic separation has revealed the basal histone synthetic profile in HEp-2 G₂ phase cells. The rate of the major histone variants, H2A.1, H2A.2, H3.1 and H3.2 decreases while that of the minor or basal histone variants, H2A.X, H2A.Z and H3.3 increases. After a 1-2 h period of chlorambucil treatment, a change in this normal G₂ pattern is induced. Basal histone synthesis continues unaffected, while, at the same time, the rate of the S phase, major H2A and H3 variants is maintained at S phase levels.

Th-187 INHIBITION OF PROTEIN SYNTHESIS AFFECTS H1 KINASE BUT NOT CHROMOSOME CONDENSATION ACTIVITY IN PIG OOCYTES

J. Motlik^a, M. Kubelka^a, J. Kalous^a, P. Guerrier^b

^aInstitute of Animal Physiology and Genetics,
Dept. of Genetics, 277 21 Libechov, Czech Republic

^bEcole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France

Protein phosphorylation was measured *in vivo* during spontaneous maturation of pig oocytes from prophase to metaphase I stage, after loading the oocytes with 32 P-orthophosphate. Western blotting with antip34^{cdc2} and anticyclin B antibodies revealed that the amount of both p34^{cdc2} and cyclin B remained nearly constant throughout this period. Histone H1 protein kinase activity, whether measured in crude extracts or after precipitation with p13-Sepharose beads, was found to increase gradually from 6 to 24 hr under our culture conditions, showing a maximum in metaphase I after 24 hrs of culture.

In addition, we found that the protein synthesis inhibitor cycloheximide, prevented protein phosphorylation, germinal vesicle breakdown (GVBD) and histone H1 kinase activation. However, this inhibition did not influence the process of chromosome condensation, what suggests that GVBD and chromosome condensation could be regulated independently. Also the levels of p34^{cdc2} and cyclin B were not influenced by cycloheximide treatment during the first meiotic division of pig oocytes.

ANALYSIS OF A TEMPERATURE-SENSITIVE YEAST MUTANT DEFECTIVE IN STATIONARY PHASE CONTROL

Th-189 T. Klade^a, L. Koller^a, K.F. Murach^a, G. Dallinger^a and M. Breitenbach^a
Department of Genetics and General Biology,
University of Salzburg, Austria

Cells of the yeast *Saccharomyces cerevisiae* enter stationary phase when meeting adverse conditions, like nutrient depletion. During this process cells acquire resistance to stress conditions and reduce metabolic activity to a basal level. Thus, cells stay viable to resume growth and proliferation once conditions are favourable again.

The *grc5* (*growth control*) mutant isolated in our laboratory suggested a function of the GRC5 gene product in stationary phase control. Interestingly, at the restrictive temperature (37°C) mutant cells lose viability on standard YPD medium very fast, but survive nitrogen starvation comparable to wild type cells. Also mutant cells arrest after two to three cell divisions with no well-defined terminal phenotype. At the permissive temperature (24°C) and when compared to wild type, *grc5* cells have an extended lag-phase and a twofold generation time. Moreover, mutant cells enter stationary phase at a cell density of about 25% of that of wild type cells without using up nutrients. We isolated four extragenic revertants of the *grc5* mutation, which complement the growth related mutant phenotypes, but which enter stationary phase at the even lower level of 10% of that of wild type cells. In the process of cloning the wild type GRC5 gene we identified two complementing DNA clones. So far, genetic and molecular analysis suggest the presence of the wild type gene on one clone and a suppressor activity on the other one. Sequence analysis of the wild type gene revealed a new yeast gene. The fact that we were able to identify several components involved with GRC5 function strongly suggests that the GRC5 gene product is part of a regulatory network involved in stationary phase control.

Th-188 THE SHORT-TERM INCREASE IN EXTRACELLULAR POTASSIUM CONCENTRATION CAUSES DNA REPLICATION ONSET IN VARIOUS NONPROLIFERATING CELLS

Y.V. Fedorov, Y.E. Yegorov, A.V. Zelenin
Engelhardt Institute of Molecular Biology,
Russian Academy of Science, Moscow, Russia

The incubation of confluent cultures of human diploid fibroblasts and 3T3 Swiss cells in the medium with 50mM K for short time induced the onset of DNA replication in a significant proportion of culture cell population. No stimulation of DNA replication was observed in the absence of serum in culture medium. The short-term increasing in extracellular concentration of potassium ions lead to onset of DNA synthesis in the cells (human diploid fibroblasts, 3T3 Swiss cells, rat smooth muscle cells) which proliferation inhibited by heparin. Also it was shown that high potassium treatment can induce aphidicolin-sensitive DNA replication in differentiated nondividing cells: mouse dorsal root ganglia neurons and in vitro differentiated myotubes formed by L6 myoblasts. All these results confirm the uniformity of nonproliferating state in the cases of high density inhibition, action of heparin and some cell type differentiation (neurons, myotubes).

THE EFFECT OF HEMIN ON HEMOPOIETIC STEM CELLS OF HEREDITARILY ANEMIC BELGRADE LABORATORY (b/b) RATS

Z. Ivanović^a, G. Jović^a, J. Marijanović^b, Z. Popović^b and P. Milenković^a

^aInstitute for Medical Research, ^bInstitute of Molecular Genetics and Genetic Engineering, Beograd, Yugoslavia

The Belgrade (b/b) rat has hereditary hypochromic myocytic anemia as the consequence of intracellular iron deficiency resulting from a genetic defect affecting transferrin bound iron transport. The studies demonstrated many disturbances in hemopoiesis of b/b rats including numerical decrease of CFU-Sd8 and CFU-GM accompanied with proliferative block of these cells in bone marrow and increase both of these cell populations in spleen. In this study, we investigated number and proliferative rate of CFU-Sd8 and number of CFU-GM in b/b rats after chronic treatment with hemin (4 weeks, three times a week, i.m., 6.5 µg/g b.w.). The results showed abrogation of proliferative block and increase in relative number of bone marrow CFU-Sd8 accompanied with a decrease of initially high number of CFU-Sd8 in spleen after hemin treatment. Treatment with hemin also increased initially low number of CFU-GM in bone marrow, indicating that hemin effect *in vivo* was not restricted to cells with capacity for erythroid differentiation. Whether the effects observed are a consequence of iron transport by heme (bypassing transferrin cycle) into cells, or by heme per se (or both), remains to be elucidated. The results suggest beneficial effect of hemin on proliferation of hemopoietic stem cells in anemic b/b rats.

Th-191

FLOW CYTOFLUOROMETRIC CHARACTERIZATION
OF *YARROWIA LIPOLYTICA* CELL CYCLE UNDER
THE INFLUENCE OF CYTOSKELETAL DRUGS

K. Augsten, I. Hönes, M. Saum, C. Lerm, and E. Unger
Dept. Molecular Cytology, Institute of Molecular
Biotechnology, Jena, Germany

The proliferation of cells is dependent on numerous factors, e.g. nutrients, physical-chemical parameters, presence of signal molecules, cell size and also different drugs. The effectiveness of such factors on the proliferation is generally dependent on the defined phase of the cell cycle.

The aim of the study was to show the influence of cytoskeletal drugs on the DNA distribution and of the morphologically definite cell cycle phases with a semisynchronously growing culture of the yeast *Yarrowia lipolytica*. The correlation between cell cycle stages and corresponding DNA content was determined. Selected drugs were: nocodazole, inducing microtubule disassembly and cytochalasin B, depolymerizing actin filaments.

The flow cyt fluorometry was used to study the DNA distribution during the cell cycle. The DNA content was determined after staining with propidium iodide and RNase digestion respectively. The different morphological phases were characterized with the fluorescence microscopy after staining with DAPI or with differential interference contrast microscopy (DIC). The DNA content is changed at a similar rate during the cells pass the G1-, S-, and G2/M-phases. Consequently, this method is suitable for quantitative characterization of the duration of cell cycle phases.

After application of nocodazole to *Y. lipolytica* the proliferation was inhibited and the number of cells with higher DNA contents subsequently increased. Similar results were obtained after addition of cytochalasin.

From these results we conclude that microtubules and actin filaments are not only involved in cell proliferation, but also in DNA replication.

CANCELLED

Th-192

MOUSE OOCYTE MATURATION: MEIOTIC
CHECKPOINTS

J. Fulka, R.M. Moor and J. Fulka

AFRC Babraham Institute, Department of
Development and Signalling Babraham,
Cambridge CB2 4 AT, England

Mouse oocytes at different stages of maturation were fused together and the onset of the next meiotic event was examined. Fusion of oocytes just undergoing germinal vesicle breakdown (GVBD) induces premature chromosome condensation (PCC) in the immature (GV) oocyte but no spindle formation. Metaphase I oocytes with spindle induce PCC together with spindle formation. Oocytes cultured for 7h (i.e. - 2h before the anaphase I onset) fused to anaphase-telophase I oocytes block the progress of meiotic maturation in advanced oocytes and induce the dispersion of chromatin on spindle. On the other hand the oocytes cultured for 8-9h but still in metaphase I respond to A-T signal and undergo this meiotic transition. The same situation is observed when A-T oocytes are fused to very early metaphase II staged oocytes. The early M II blocks A-T I transition; the late M II responds to A-T I signal and undergoes the A-T II transition. That the chromosomes are responsible for this control is demonstrated by fusion of chemically enucleated oocytes to late M I oocytes; here the onset of A-T I is not influenced. Moreover, these oocytes can be therefore activated. Moreover, the immature (GV) oocytes are unable to recognize the replicating DNA and undergo germinal vesicle breakdown.

Th-194

SERUM ALBUMIN PROTECTS THE
BIOLOGICAL ACTIVITY OF ENZYMES DURING
HEAT STRESS

E.C.Nedea, M.V.Leabu

Department of Cell Biology and Histology,
"Carol Davila" University of Medicine and Pharmacy, Bucharest,
Romania

Serum albumin is known to protect the biologically active proteins against stress conditions such as changes in pH, ionic strength and temperature variations during freezing and thawing.

This study analyzed the ability of BSA to protect various enzymatic activities during heat stress. Enzymatic activities were measured for lactate dehydrogenase (LDH) at 340 nm, with NADH as cosubstrate and for catalase at 240 nm, with H₂O₂ as substrate before and after heat shock, with or without BSA.

The LDH activity after 15 minutes heating without BSA at 55 and 60 °C was decreased by 32 and 70 % respectively, from the control value measured at 25 °C. When BSA was added, the LDH activity was unchanged after heating at 55 °C and decreased by 37 % at 60 °C. The peroxidative activity of catalase after 60 minutes at 42 °C without BSA was reduced by 28 % from control value, while in the presence of BSA the decrease was by 11 % only. BSA significantly diminished catalase aggregation in a concentration-dependent manner.

The results prove the ability of BSA to protect the biologically active proteins during heat stress and suggest a mechanism in which a possible inhibition of protein aggregation is involved.

CANCELLED

Th-196 PIG PLASMA MODULATES CELL CYCLE KINETICS BUT NOT THE BASELINE FREQUENCY OF SISTER CHROMATIDS EXCHANGES IN HUMAN LYMPHOCYTES

M.L. Larramendy and M.A. Reigosa

Laboratorio de Citoogenética y Cátedra de Citología, Facultad de Ciencias Naturales y Museo, La Plata, Argentina

The effect of human and pig plasma on the baseline frequency of sister chromatid exchanges (SCEs) in human and porcine plasma leukocyte cultures (PLC) was studied. Human PLC, but not pig PLC, showed at least a two-fold increase in SCE frequency over whole blood culture (WBC) values. Addition of pig plasma to human PLC and human plasma to pig PLC did not modify the baseline SCE frequencies. In both human and porcine cultures, cell proliferation was slower in PLC than in WBC. The addition of pig plasma to human PLC, but not the incorporation of human plasma in pig PLC, accelerated the cell cycle progression of lymphocytes. With 10% pig plasma in the leukocyte culture medium, lymphocyte proliferatin was similar to that in WBC. Smaller concentrations of pig plasma rendered cell cycle progression intermediate between the basal PLC and WBC values. Exchanging fetal calf serum for human AB serum in human PLC did not affect the cell cycle kinetics of lymphocytes but did decrease their basline SCE frequency.

Th-197 PHASE STEPPING INTERFERENCE MICROSCOPY IN CELL BEHAVIOUR

D. Zicha^{a,b}, G.A. Dunn^a

^aMRC Muscle and Cell Motility Unit, The Randall Institute, King's College London, 26-29 Drury Lane, London WC2B 5RL, UK; ^bInstitute of Molecular Genetics, Academy of Sciences of the Czech Republic, Fleming Place 2, 166 37 Prague 6, CZ

Major advantage of Phase Stepping Interference Microscopy (PSIM) is well defined interpretation of the image (Dunn and Zicha, 1994). Two-dimensional distribution of light intensity reveals the distribution of non-aqueous mass within cells. The application of PSIM in cell behaviour has been made possible by the progress in computer image processing. The compatibility of PSIM with automated image processing makes the technique uniquely suitable for cell behaviour studies because it generates automatically reliable descriptors of cell behaviour about large numbers of cells.

The Jamin-Lebedeff microscope adapted for PSIM demonstrated the sensitivity of the technique, by exact detection of the growth of single cells. The Horn microscope using the technique at lower magnification proved reliable mass detection of cells showing social behaviour in subconfluent culture.

Dunn, G.A. and Zicha, D. (1994) Using interference microscopy to study cell behaviour. In *Handbook of Cell Biology*, J.E.Celis (ed) Academic Press Inc., (in press).

Th-198 CONFOCAL SCANNING IMAGING SYSTEM IN THE PROCESS OF INTRACELLULAR TRANSPORT AND CONFORMATIONAL CHANGES OF VIRUS STRUCTURAL PROTEINS

F. Čiampor

Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Virus structural proteins provide an excellent model for studying transport and conformational changes during the transport and functional dynamics of eucaryotic proteins as well as their role in the biology of viral replication and assembly.

The confocal scanning imaging system techniques considered in the process of intracellular transport of virus structural proteins include:

a/ Monoclonal or polyclonal antibodies produced against virus structural proteins and characterized which conformational or trans-membrane part of the molecule can be recognized.

b/ Immunocytochemical, immunogold silver approaches that make it possible to specifically label intracellular and cell surface molecules. Confocal scanning imaging system in the study of protein intracellular transport offers several advantages over conventional light microscopy and electron microscopy. The optical sectioning eliminates the artifacts of physical sectioning and specimens are scanned in vertical and horizontal plane. Fixation plays a crucial role in epitope and paratope interaction, mainly in a very distinct conformational changes of transported molecules.

Th-199 EXPRESSION OF CARTILAGE SPECIFIC LINK PROTEIN AND CARTILAGE MATRIX PROTEIN GENES IN DIFFERENTIATING CHONDROCYTES

Csand Bachrati^{a,b}, Beatrice Dozin^a, Ibolya Kiss^b and Ranieri Cancedda^a

^aLaboratorio di Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy

^bInstitute of Biochemistry, Biological Research Centre of Hungarian Academy of Sciences, Szeged, Hungary

Expression of cartilage specific link protein (LP) and cartilage matrix protein (CMP) genes were monitored in an *in vitro* cell culture system that was proved to be an appropriate model for studying the *in vivo* process of the enchondral bone formation in the developing limb bud.

Chondrocytes when enzymatically dissociated from stage H.H. 28-30 chicken embryo tibiae and cultured as adherent cells assume a fibroblast-like morphology, and express type I collagen. When these dedifferentiated cells are transferred into suspension culture, they rapidly aggregate, and within 2 weeks undergo a transient proliferative phase (stage I), characterized by synthesis of type II collagen. Within 4 weeks they reach final maturation as hypertrophic chondrocytes (stage II) with characteristic expression of type X collagen. When the hypertrophic chondrocytes are further cultured as adherent in the presence of ascorbic acid they differentiate towards an osteoblast-like phenotype.

We followed the expression of the LP and the CMP genes at the RNA level by Northern blot hybridization and compared it with that of collagens $\alpha 1(I)$, $\alpha 1(II)$ and $\alpha 1(X)$. The LP and CMP genes are switched on in parallel to the transient stage I phase. The level of expression remains high also in the stage II, but disappears when the cells are reattached. Therefore the expression of these two genes is a useful marker for staging chondrocyte differentiation process.

Th-201 ESSENTIAL ROLE FOR N-LINKED CARBOHYDRATES REVEALED IN MUTANT MICE LACKING THE GOLGI TRANSFERASE, N-ACETYLGLUCOSAMINYLTRANSFERASE I.

Pamela Stanley and Ella Ioffe

Dept. Cell Biology, Albert Einstein College Medicine, New York, NY 10461

The glycosyltransferase termed N-acetylglucosaminyltransferase I (GlcNAc-TI) initiates the synthesis of hybrid and complex N-linked carbohydrates in mammals. CHO mutants lacking GlcNAc-TI(Lec1 and 15B) have no apparent growth defects. Both classes of N-linked carbohydrates are thus dispensable for cultured cells. We have now shown that, by contrast, GlcNAc-TI activity is absolutely necessary for mouse development. Mice that carry two disrupted *Mgat-1* alleles generated by homologous recombination in ES cells, die at mid-gestation. At embryonic day 9.5, *Mgat-1* -/- mice have no detectable GlcNAc-TI activity nor complex carbohydrates, though they have normal levels of b(1,4)Gal-T. By day 10.5, -/- embryos are deteriorating and none are recovered after day 11.5. Heterozygotes are however not detectably different from mice with two functional copies of the *Mgat-1* gene. ES cell lines that possess two disrupted *Mgat-1* alleles were generated by selection in G418. Independent isolates are now being studied for their abilities to differentiate in culture and to contribute to different tissues in chimeras.

Th-200 EMBRYOGENESIS OF CUCUMIS SATIVUS L.

M.Kalina, V.Duplij, V.Sidorov

Institute of Cell Biology & Genetic Engineering, Zabolotnogo 148, Kiev-DSP-22, 252022, Ukraine.

The aim of our work was to find the conditions for plant regeneration from mesophyll protoplasts as well as from leaf and cotyledon sections of cucumber.

Cotyledon and leaf segments were placed onto the induction medium containing various combinations of auxins (2,4-D, NAA, IAA) and cytokinins (kinetin, BAP, 2iP, zeatin). After three weeks of cultivation in darkness explants were transferred onto the same medium without growth regulators. Embryogenesis was observed after 4-6 weeks of cultivation in light. High regeneration efficiency for leaf sections and satisfactory for cotyledon segments was provided by medium containing 3 mg/l 2,4-D and 3 mg/l kinetin. A number of growth regulator combinations was tested for mesophyll protoplast derived calli. High regeneration frequency (about 50%) was obtained on the same induction medium.

These data have supported our suggestion that conditions of embryogenesis have to be the similar both for cotyledon or leaf explants and for mesophyll protoplasts.

DNA SEQUENCING TECHNOLOGY TODAY AND TOMORROW

W. Ansorge, D. Grothues, H. Erfle, N. Hewitt, T. Kupp, C. Schweger, C. Sensen, H. Voss, S. Wiemann and J. Zimmermann

Th-202

European Molecular Biology Laboratory, Meyerhofstr. 1, 6900 Heidelberg, F.R.G.

On-line automated DNA sequencing technology with a throughput of up to 100 kilobases of sequence per device per day, representing about ten-fold improvement over the current techniques, is being developed. The output of the gel-based sequencing system is improved in several ways: (1) increasing the number of clones run simultaneously on a gel, achieved through design of new detectors allowing miniaturisation of the sample tracks, and by running simultaneously clones labelled with two or more dyes; (2) increasing the speed with which the bands migrate in the gel, using ultrathin gels allowing higher voltage (J. Stegemann et al., 1991, NAR, 19, p. 675) or by using new gel matrices; (3) reusing the gels several times; and (4) increasing the number of bases resolved in one run by increasing the separation distance in the gel, optimizing template preparation, sequencing reactions and introducing the new internal label fluorescent dNTPs technique. About 1000 bases are now resolved routinely in a single sequencing run on the A.L.F. DNA sequencer (Pharmacia), using fluorescein-15-dATP T7 DNA polymerase, 5% Hydrolink gel, detection distance 50 cm. The efficiency is further improved by direct sequencing of large vectors, e.g. cosmids, avoiding subcloning of the insert. The on-line sequence throughput of the system will be further increased by expanding the number of dyes run simultaneously to three or more, approaching the potential capacity of the multiplex off-line method.

CAN PROTEIN BODIES FUNCTION IN DEFENCE
REACTIONS OF PLANT CELLS?

Th-203 J. Hrib^a, R. Janisch^b and B. Vooková^a

^aInstitute of Plant Genetics, Slovak Academy of Sciences, 950 07 Nitra, Slovakia
^bDepartment of Biology, Faculty of Medicine, Masaryk University, 662 43 Brno, Czech Republic

A simple technique of dual culture was used in an *in vitro* study of defence reactions of the embryo, the embryo with the megagametophyte and the megagametophyte of the European black pine (*Pinus nigra* Arn.) to a tester, the basidiomycete *Phaeolus schweinitzii* (Fr.). On the agar medium B-25 with naphthaleneacetic acid (5 mg/L) and benzylaminopurine (0.1 mg/L), the defence reaction effective at a long distance was stronger in the embryo than in the megagametophyte. Total inhibition of the mycelial growth of the tester resulted from either a direct contact or small distance (up to 1 mm) between the megagametophyte and tester. This type of defence reaction shown by the megagametophyte was remarkable and can be accounted for by the presence of high molecular substances, storage proteins. These protein bodies contain globulins rich in glutamine and our experiments showed that L-glutamine inhibited mycelial growth of *L. schweinitzii*. The bodies probably became involved in the defence reaction after their hydrolysis. The degraded protein bodies in megagametophyte parenchyma cells were studied by freeze-etching.

THE FUNCTION OF HEAT-SHOCK PROTEINS

Th-204

A. Venetianer, M. Pirity and A. Hevér-Szabó, Inst. of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

We earlier demonstrated that hsp68 is defecitarily induced upon stress in the glucocorticoid-resistant rat hepatoma clone 2 cells, but is strongly activated in the glucocorticoid-sensitive Faza 967 cells from which it was derived. We used the clone 2 cells to address the questions of whether hsp68 is specifically involved in the development of thermotolerance and/or thermoresistance. Our experiments show that clone 2 cells were not protected from the killing effect of heat by pre-treatment with sodium arsenite, whereas Faza 967 cells were. These results strongly suggest a role of hsp68 in the development of thermotolerance in hepatoma cells. Stable heat-resistant variants of clone 2 cells were also isolated, where an increased basal expression of several hsp's was observed together with the (at least partial) restoration of the heat-inducibility of hsp68 indicating that several hsp's are needed to protect the cells at high temperature. The heat-resistant hepatomas became also resistant to several anticancer drugs. The multidrug resistance of the hepatoma variants correlates with the overexpression of the P-glycoprotein. Our results showing that severely stressed hepatoma cells overexpressed the mdr gene(s) raise the possibility that P-gp may participate in the protection against stress.

MASS SPECTROMETRY AND SEQUENCE DATABASES FOR THE IDENTIFICATION OF PROTEINS

Th-205 M. Mann

European Molecular Biology Laboratory, Heidelberg, Germany

Mass spectrometric data - which today is orders of magnitude easier to obtain compared to even five years ago - is correlated with the sequences in the databases. We have investigated three kinds of mass spectrometric information in this context: (i) the complete molecular weight of the protein, (ii) the masses of peptides obtained by a sequence specific protease or chemical reagent and (iii) the mass of a single peptide combined with partial sequence information provided by MS/MS data or by Edman degradation. The three methods are of differing generality, with (i) being applicable only in a few specialized cases and (iii) being the most powerful and general (1, 2).

The program used in this investigation (Peptide-Search) was written for the Macintosh computer and features very flexible searches (limit digestion or consideration of one or two missed cleavages), many specific proteases and the ability to define rules for digestion. This flexibility is provided by very fast "on the fly" digestion which also allows search by partial sequence information on the same file. As an example, search times on SWISSPROT (ca. 30 000 proteins) are less than 20 second on Quadra type Macintosh computer.

Experimental results used to illustrate the search specificities were taken with a Bruker REFLEX mass spectrometer using Matrix Assisted Laser Desorption / Ionization (MALDI) and with a Sciex API III triple quadrupole electrospray mass spectrometer.

Applications include the rapid and sensitive identification of known proteins from one or two dimensional gels and the identification of protein - protein interactions.

TOWARDS A COMPLETE HUMAN PROTEIN INDEX

Th-206 J. E. Celis^a, H. H. Rasmussen^a, H. Leffers^a, K. Dejgaard^a, P. Madsen^a, B. Honoré^a, E. Olsen^a, B. Gesser^a, P. Gromop^a, H. J. Hoffmann^a, M. Nielsen^a, J. Van Damme^b, M. Puype^b, and J. Vandekerckhove^b

^aInstitute of Medical Biochemistry and Danish Centre for Human Genome Research, University of Aarhus, Denmark

^bLaboratory of Physiological Chemistry, State University of Ghent, Ghent, Belgium

Complex patterns of protein expression - which often comprise up to 3,000 proteins or more - can be analyzed by computer-aided two-dimensional (2-D) gel electrophoresis, and the information (mainly qualitative at present) stored in databases that aim at integrating protein (identity, cellular localization, levels under various physiological conditions, coregulated proteins, function, partial protein sequences, etc.) and DNA mapping and sequencing information (chromosome location, regulatory elements, etc.). Currently, the Aarhus keratinocyte database lists nearly 3,500 cellular and secreted proteins, a number that is close to the estimated 5,000 - 5,500 proteins thought to be present in this cell type. Of these, about 900 have been identified by using a plethora of technology that includes 2-D gel immunoblotting, comigration with purified human proteins, microsequencing, mass spectrometry, and expression of cDNAs in the vaccinia virus system. So far, nearly 400 proteins recorded in the database have been microsequenced, and a substantial number correspond to hitherto unknown proteins that are amenable to cDNA cloning. Current status of the database and strategies aimed at achieving a complete human protein index will be described.

**ASSEMBLY OF RETROVIRAL CAPSIDS IN
EUKARYOTIC AND PROKARYOTIC CELLS
Th-207 AND IN VITRO**

Tomas Rumí

Department of Microbiology and Biochemistry,
Institute of Chemical Technology, Prague, Czech
Republic

Retrovirus assembly involves transport of precursor macromolecules to a single point in the cytoplasm or at the plasma membrane according to the morphogenetic type of virus. Capsids of Mason-Pfizer monkey virus, as other type-D retroviruses, are preassembled within the cytoplasm prior to transport to the cell membrane.

Capsid precursor protein of M-PMV has been expressed in yeast and bacterial cells. In *Saccharomyces cerevisiae*, the gag gene was expressed using vector in which transcription is driven from the methionine promoter. Assembly of particles of similar shape and size to those in mammalian cells was observed in yeast by electron microscopy. Capsid-like structures accumulated in a distinct sites of cytoplasm. Similar particles corresponding to fully and aberrantly assembled capsids were observed also in *Escherichia coli* expressing M-PMV gag gene under the control of the phage T7 promoter. All the capsid like structures were localized within the inclusion bodies that formed at poles of the cells. The inclusion bodies were solubilized completely in urea and following renaturation, under specific conditions, *in vitro* assembly of capsid-like structures with icosahedral symmetry was observed.

**CLEAVAGE OF HOST CELL PROTEINS BY HIV-1
PROTEASE. IMPLICATIONS FOR CYTOPATHOGENESIS
Th-208**

Robert L. Shoeman, Claudia Hüttermann,
Elfriede Mothes and Peter Traub

Max-Planck-Institut für Zellbiologie,
68526 Ladenburg, Germany

Retroviruses encode an aspartyl protease that is responsible and required for the ordered cleavage of the gag and gag-pol polyproteins during the maturation process that leads to the formation of infectious viral particles. Several research groups have found that the HIV-1 protease can cleave, in addition to the viral polyproteins, a variety of important host cell proteins, including transcription factors (NF-kappaB precursor), extracellular matrix proteins (fibronectin), calmodulin and diverse elements of the cytoskeleton (i.e., myosin and actin; microtubule associated proteins; spectrin; intermediate filament proteins, including vimentin, desmin and glial fibrillary acidic protein; etc.) While most of these studies have been performed *in vitro* with purified components, the cleavage of vimentin, actin, desmin and tropomyosin have been demonstrated *in situ* and *in vivo* and the cleavage of vimentin and actin also occurs in HIV-1 infected cells. We have shown that tissue culture cells treated with HIV-1 protease demonstrate a number of cytoskeletal and nuclear abnormalities that are reminiscent of HIV-1 infected cells. We propose that cleavage of host cell proteins plays at least two different roles in infected cells. The first is direct cell killing by extensive alterations of important cellular elements, such as the cytoskeleton and actin/myosin-based motility systems. The second is alteration of host cell gene expression, resulting in either cell death or abnormal cell proliferation, in keeping with models that propose a global role in gene expression for the cytoskeleton in general and IFs in particular.

**CELLULAR LOCALIZATION OF M-PMV
PROTEINASE IN E. COLI AND ITS
DIFFERENT ACTIVE FORMS**

Th-209 I. Pichová^a, O. Hrušková-Heidingsfel-dová^a, A. b Zábranský^a, M. Andreánský^b, E. Hunter^b and T. Rumí^a

^aInstitute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague; ^bUniversity of Alabama at Birmingham, Alabama, USA

Proteinase of Mason-Pfizer Monkey Virus (M-PMV), primate retrovirus, is translated as a part of Gag-Pro 95 kDa polyprotein.

We expressed M-PMV proteinase in *E. coli* in T7 polymerase/promoter system from construct containing a truncated pro gene and leading to a 26 kDa proteinase precursor. This hydrophobic precursor is preferentially accumulated in the cytoplasmic inclusions but about 40% of the precursor is localized in cell cytoplasm. We used series of effective inhibitors to protect *E. coli* during the expression of PR, which causes a damage of bacterial cells. The localization and the yield of recombinant product were checked. The selfprocessing of proteinase isolated from inclusion bodies occurs stepwise. The initial product is 17 kDa active "precursor" which is further cleaved to provide 10 kDa mature product. The stability of 17 and 10 kDa forms highly depends on their concentrations and a combined effect of pH and ionic strength. The control and stepwise activation of the proteinase might be a regulatory phenomenon influencing the retroviral replication.