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The Origin of Metazoan Complexity: Porifera as Integrated Animals[1]

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SYNOPSIS. Sponges [Porifera] are the phylogenetically oldest metazoan phylum still extant today; they share the closest relationship with the hypothetical common metazoan ancestor, the Urmetazoa. During the past 8 years cDNAs coding for proteins involved in cell-cell- and cell-tissue interaction have been cloned from sponges, primarily from *Suberites domuncula* and *Geodia cydonium* and their functions have been studied *in vivo* as well as *in vitro*. Also, characteristic elements of the extracellular matrix have been identified and cloned. Those data confirmed that all metazoan phyla originate from one ancestor, the Urmetazoa. The existence of cell adhesion molecules allowed the emergence of a colonial organism. However, for the next higher stage in evolution, individuation, two further innovations had to be formed: the immune- and the apoptotic system. Major defense pathways/molecules to prevent adverse effects against microbes/parasites have been identified in sponges. Furthermore, key molecules of the apoptotic pathway(s), *e.g.*, the pro-apoptotic molecule comprising two death domains, the executing enzyme caspases, as well as the anti-apoptotic/cell survival proteins belonging to the Bcl-2 family have been identified and cloned from sponges. Based on these results—primarily obtained through a molecular biological approach—it is concluded that cell-cell- and cell-matrix adhesion systems were required for the transition to a *colonial stage* of organization, while the development of an immune system as well as of apoptotic processes were prerequisites for reaching the *integrated stage*. As the latter stage already exists in sponges, it is therefore likely that the hypothetical ancestor, the Urmetazoa, was also an "integrated colony."

INTRODUCTION

The origin of Metazoa is to some extent still enigmatic despite the progress that has been achieved in the last years by molecular studies. The evolution of the Metazoa from unicellular/colonial organisms occurred some 1,300–600 Myr ago in the pre-Ediacaran period (Conway Morris, 1998). Morphological contributions to understanding of the transitional stages to the Metazoa suggest a colonial origin of Metazoa (see Dewel, 2000). This view implies that, based on the Beklemishev's cycles of duplication and individuation (Beklemishev, 1969), after duplication of an individual and the formation of a colony this entity has to undergo individuation again. It has been pointed out that two such cycles were necessary in early evolution for the emergence of Metazoa: first the transition to multicellular organisms, with the sponge grade of organization, and second the change to the modularized ancestor of the Bilateria (Dewel, 2000).

Recent phylogenies based on rDNA have suggested, that the Metazoa are polyphyletic. The proposal suggested that the Porifera/Cnidaria evolved separately from the Triploblasts; both having originated independently through aggregation of protists belonging to two different lineages (Christen *et al.*, 1991). This view is based on the early idea that sponges (Porifera) are grouped with the Protozoa (Spencer, 1864, p. 302). Later, ontogenetic evidence provided the basis for sponges to be considered as metazoans (Haeckel, 1896, p. 18). However, until recently it was generally accepted that the choanoflagellates were the sister group to Metazoa (see Nielsen, 2001). Even though sponge choanocytes are similar to choanoflagellates and are composed of a single flagellum, surrounded by a microvillar collar, the long-standing view of a homology of these types of flagella/cilia (Kent, 1881; Lackey, 1959) could not be substantiated (Karprov and Efremova, 1994). Now, based on the study with protein molecules, Fungi are regarded as the nearest-neighbor kingdom of the Metazoa (Schütze *et al.*, 1999), a conclusion which is supported by others (Baldauf *et al.*, 2000).

During the past 10 years unequivocal support for the monophyly of metazoans has been presented (Müller, 1995) from molecular studies, from analysis of specific cell adhesion molecules and their receptors as well as of the extracellular matrix in sponges, and especially by combination of these data with morphological studies (see Dewel, 2000). The assumption that all metazoan phyla are of monophyletic origin has been widely accepted (see Borchiellini *et al.*, 2001). For the molecular biological analyses the two demosponges *Suberites domuncula* and *Geodia cydonium* have been used. The facts compiled also imply that the ancestor of all metazoans was a sponge-like organism, which I termed Urmetazoa (Müller, 2001). This new step in understanding of the basal animal phylogeny is the platform for answering the next pressing question of the origin of individuation in Metazoa, again regarding Porifera as living fossils, descendants of a colonial ancestor. The step of individuation taken by sponges was also a prerequisite for the further progress in evolution of the Porifera to the level of Cnidaria (Dewel, 2000; Fig. 1).

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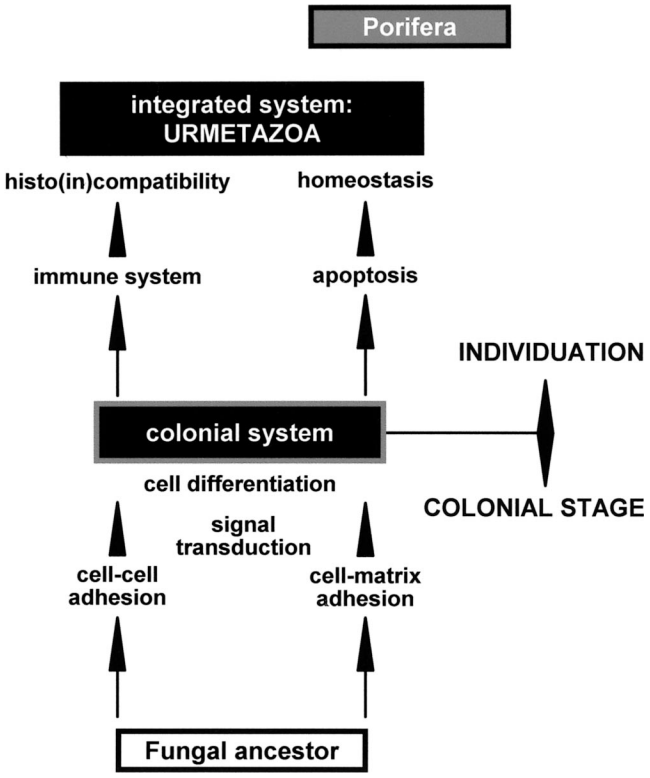

FIG. 1. Hypothetical steps towards the evolution to the Urmetazoa with the Porifera as the next closest taxon. Adhesion molecules were required to allow the transition from a fungal-like ancestor to a colonial system, a stage which made the further development of immune- and apoptotic systems possible that led to the evolution of the Urmetazoa, as an integrated system.

In the following review I present a summary of the recent achievements in the understanding of principles of individuation in sponges, based on protein sequences functioning in the immune response and apoptosis, two processes which play central roles in the maintenance of development and homeostasis of metazoans.

METAZOAN ORIGIN: MONOPHYLY

The adhesion molecules in sponges provide solid grounds for the view that all metazoan animals originated from one ancestor, the Urmetazoa (reviewed in Müller, 2001 and 2003). These molecules were found to represent major metazoan autapomorphies (see Müller, 1995 and 1997).

Adhesion molecules

Already since their first use by Wilson (1907) sponges have been a traditional model for studies of cell-cell and cell-matrix adhesion (reviewed in Burger and Jumblatt, 1977; Müller, 1982). Primarily the two marine demosponges, *Microciona prolifera* and *G. cydonium*, have been the most thoroughly studied species. In 1973 two groups succeeded in isolating and purifying both from *M. prolifera* (Henkart *et al.*, 1973) and *G. cydonium* (Müller and Zahn, 1973) the first extracellular particle, termed an aggregation factor (AF), which promotes the species-specific aggregation of sponge cells.

The *G. cydonium* AF is a complex particle, composed of several polypeptides. Three proteins associated with the AF from *G. cydonium* were identified in detail; a galectin, a 36 kDa putative AF as well as an 86 kDa AF-associated polypeptide. In addition, the proteoglycan-like core structure of the AF has been characterized from *M. prolifera*. The *G. cydonium galectin* was cloned; sequence analysis revealed that those aa residues which are involved in mammals in binding of galectins to galactose are conserved in the sponge sequence (Pfeifer *et al.*, 1993). This observation was taken as evidence in support of the monophyly of Metazoa (Müller, 1995). The galectin links the AF-complex to the membrane-associated aggregation receptor (AR) (Wagner-Hülsmann *et al.*, 1996). The *36 kDa putative AF* was recently cloned (Schütze *et al.*, 2001*a*). Its deduced aa sequence displays in the N-terminal portion high similarity to amphiphysin/BIN1 sequences found in Protostomia and Deuterostomia. In addition, an *86 kDa AF-associated polypeptide* has been identified, whose predicted protein comprises nine short consensus repeats (SCR) (Müller, 2003). The *core structure* of the AF had been identified in the AF-complex from *M. prolifera* as a polymorphic proteoglycan-like molecule (Fernandez-Busquets *et al.*, 1996).

Using the *G. cydonium* model sponge the *putative aggregation receptor* (AR) was cloned (Blumbach *et al.*, 1998). It comprises fourteen scavenger receptor cysteine-rich (SRCR) domains, six SCR repeats, a C-terminal transmembrane domain and a cytoplasmic tail. Competition experiments using recombinant AR or antibodies raised against this receptor, suggested that the adhesion molecule present in the enriched AF binds to the AR. In addition, previous experiments also indicated that the strength of binding of the AF to the cell surface AR is augmented by galectin (Wagner-Hülsmann, 1996).

Extracellular matrix molecules

In sponges the space between the external pinacoderm and the internal choanoderm, the mesohyl, does not comprise a homogenous ground substance. It is composed, in addition to galectin, of the following main elements: collagen, fibronectin-like molecules, and a minor component, dermatopontin, was also recently identified, (Schütze *et al.*, 2001*b*). These polypeptides form the extracellular matrix (ECM) which provides the platform for specific cell adhesion via the integrin receptor, as well as for signal transduction and cell growth. As an example, it has been summarized that in demosponges several cells are involved in spicule formation (Uriz *et al.*, 2000). This process requires a series of complex pathways in which also the expression of silica-responsible genes is involved (Krasko *et al.*, 2000 and 2002).

Collagen is an autapomorphic molecule that is present only in the Metazoa. In contrast to higher meta-

zoan phyla, which contain approximately 20 different types of collagen, in sponges only two groups of collagen molecules have been identified, the fibrillar collagen and the type IV-related collagen (reviewed in Garrone, 1998). Exposito and Garrone (1990) were the first to sequence a collagen cDNA from a sponge. Recently, it was shown that cells of *S. domuncula* express a collagen gene in response to the growth factor myotrophin (Schröder *et al.*, 2000). The cDNA for *S. domuncula* collagen was isolated; the deduced aa sequence shows that the collagenous internal domain is rather short with only 24 G-x-y collagen triplets (Schröder *et al.*, 2000).

A further major component of the ECM is *fibronectin*. Evidence has been presented, suggesting that sponges also contain fibronectin. In 1981 Labat-Robert and colleagues described a protein in sponges, which cross-reacted with antibodies raised against vertebrate fibronectin (Labat-Robert *et al.*, 1981). During our search for fibronectin we were able to demonstrate that in *G. cydonium*, there are protein(s) that cross-react immunologically with human anti-fibronectin antiserum (Müller, 1997). A subsequent screening for the respective cDNA revealed a protein which consists of three putative modules; a fibronectin module type-III, a SRCR unit and a SCR repeat; this sponge protein was called a ''multiadhesive protein'' (Pahler *et al.*, 1998*a*).

One major class of receptors which interact with the ECM are the *integrin* receptors, membrane-anchored heterodimer receptors composed of α- and ß-subunits. After the identification of collagen in the ECM subsequent screening produced the integrin receptors in the marine sponges *G. cydonium* (Pancer *et al.*, 1997) and *S. domuncula* (Wimmer *et al.*, 1999).

One additional sponge membrane receptor that should also be mentioned here, is the *receptor tyrosine kinase* (RTK). RTKs are restricted to the Metazoa. The first RTK from lower metazoa was identified and cloned from *G. cydonium* (reviewed in Müller and Schäcke, 1996). As schematically outlined in Figure 2 (GC-RTK), the deduced polypeptide sequence comprises: *(i)* the extracellular part with a Pro/Ser/Thr-rich region, and two complete immunoglobulin-like (Ig-like) domains, *(ii)* the transmembrane domain, *(iii)* the juxtamembrane region and *(iv)* the catalytic tyrosine (TK)-domain. A ligand for this RTK, a mucus-like protein, was also identified (Schütze *et al.*, 2001*b*).

Metazoan-fungal relationship

Using protein DNA sequences from sponges, especially those from adhesion molecules, the monophyletic origin of the Metazoa was established (Müller *et al.*, 2001*a*). Using those molecules which function in signal transduction, growth control and defense, a common ancestry with Fungi was established (Schütze *et al.*, 1999; Müller *et al.*, 2001*a*); Figure 1. Already before such a relationship could be proposed (Borchiellini *et al.*, 1998). The existence of cell-cell- and cell-matrix adhesion molecules was used as evidence

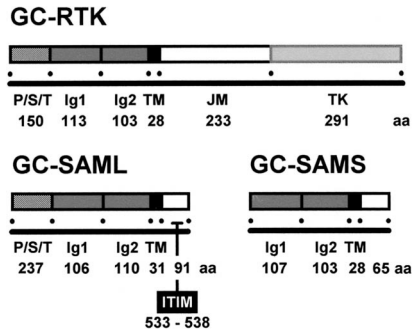

FIG. 2. Molecules from *G. cydonium* comprising Ig-like domains. Structure of the receptor tyrosine kinase (GC-RTK), as well as of the sponge adhesion molecules (SAM), the long form GC-SAML, and the short form GC-SAMS, from *G. cydonium*. The building blocks are: Pro-Ser-Thr(P/S/T)-rich domain, Ig-like domains 1 (Ig 1) and 2 (Ig 2), transmembrane domain (TM), juxtamembrane region (JM) and TK-domain (TK). The length of the stretches of the respective deduced aa domains are given. The position of the ITIM-motif in the cytoplasmic region of the *G. cydonium* GC-SAML (spanning aa_{533} to aa_{538} of the polypeptide) is marked.

of the evolution from the COLONIAL STAGE to an INTEGRATED STAGE, the step towards individuation (Fig. 1).

METAZOAN INDIVIDUALITY: IMMUNE MOLECULES

Adhesion of cells is the basic property and prerequisite for a functional immune system. Therefore, it can be assumed that during evolution elements or molecules developed which became functional not only as adhesion molecules but also as elements of the immune response. One well known example is the immunoglobulin (Ig-) domains which are building blocks of polypeptides that participate in cell adhesion, muscle contraction, and immune defense (Nezlin, 1998). Later in evolution these domains served as immune molecules.

The early metazoans, the hypothetical Urmetazoa, lived in an aquatic environment and consequently were exposed to a massive load of both pro- and eukaryotic organisms trying to invade and destruct them. It is amazing that sponges have the capacity to process their own volume of water every 5 seconds in order to extract edible material (Vogel, 1977); this fact supports the notion that they are exposed to a huge number of bacteria and also viruses present in the seawater (see Gonzales and Moran, 1997). To cope with these threats sponges have developed an efficient chemical defense system (Proksch, 1994) as well as humoral and cellular defense mechanisms (Müller *et al.*, 1999*a*). Studies on the immune system in sponges have been performed with the focus on the mechanisms by which *(i)* these animals react against microbes/parasites and *(ii)* respond to non-syngeneic tissue.

In recent studies it has been reported that there are pathways which control fusion and rejection during histo-(in)compatibility reaction in the Porifera (Müller *et al.*, 1999*a*, 2001*b*). Although this had been expected from the precise historecognition reactions that were

described on tissue level (see: Hildemann *et al.*, 1979 and 1980), it was very surprising to discover that key molecules involved in allo/auto-immunity in sponges share high sequence and functional similarity with those molecules which had been found to control historecognition in deuterostomes. Among those are the molecules comprising polymorphic Ig-like domains (present in the sponge adhesion molecules [SAMs]), the allograft inflammatory factor (a sponge cytokine) as well as the (2–5)A system (control of infection) (see below), whose existence had not been reported in protostomes (Gamulin *et al.*, 2000; Müller *et al.*, 2001*a*). This fact, that sponges have molecules/pathways in common only with deuterostomes *(i)* strongly supports the monophyly of Metazoa, *(ii)* underscores that the degree of individuality of sponge species is high and *(iii)* suggests that sponges might/will become model organisms to understand the origin of vertebrate immunity and diseases connected with it.

METAZOAN INDIVIDUALITY: APOPTOTIC MOLECULE

Apoptosis in sponges

Until recently (Wiens *et al.*, 2000*a, b*) it was proposed that the physiological cell death is restricted to multicellular organisms, which have separate germ and somatic cells (Vaux *et al.*, 1994). Originally it was suggested to divide the process of physiological cell death into *(a)* ''programmed cell death,'' describing the developmentally regulated elimination of specific cells during embryogenesis (Lockshin and Williams, 1964), and *(b)* ''apoptosis'', describing morphological changes of dying cells (Kerr *et al.*, 1972). At present, these terms are used interchangeably; therefore, we use the term apoptosis. In the last three years it has become apparent that apoptosis is not restricted to metazoans that have separate cell lines, but came about during the transition from the common ancestor of all metazoan phyla to the phylogenetically oldest metazoan taxon, the Porifera (reviewed in Müller *et al.*, 1998).

Two lines of evidence led us to assume that sponges are also provided with complex apoptotic pathways. In 1992 Pfeifer and others found that a factor could be identified in xenografts from *G. cydonium* that cross-reacted immunologically with an antibody raised against a mammalian tumor necrosis factor (TNF). The M_r was determined to be 30 kDa hinting at a relationship to the mammalian TNF. Furthermore, it was shown that sponge cells have a high level of telomerase activity, when they are present in the state of cell-cell contact [both in intact organism and in primmorphs] (Koziol *et al.*, 1998). Consequently we postulated that, in order to maintain a defined ''Bauplan,'' sponge cells in tissue organization must undergo apoptosis (Wagner *et al.*, 1998). Recently, we could identify in sponges homeobox genes, *e.g.*, a LIM/homeobox encoding protein, which are involved in organogenesis in higher metazoan phyla (Wiens *et al.*, submitted). However, in spite of intense efforts, the gene encoding the potential TNF as well as the receptor interacting in mammalian systems, the TNF-receptor, were only recently cloned. The first potential gene involved in apoptosis of sponge cells, the *MA-3* gene from *S. domuncula* was identified (Wagner *et al.*, 1998); the corresponding mouse *MA-3* cDNA is assumed to encode an apoptotic molecule (Shibahara *et al.*, 1995). Subsequently both pro- and anti-apoptotic proteins have been cloned from both *S. domuncula* and *G. cydonium*, and their functions have been analyzed to some extent.

Metazoan pro-apoptotic molecules

As the most promising segment to screen for a pro-apoptotic molecule, we selected the death domain part which is found in the mammalian apoptosis controlling proteins Fas, tumor necrosis factor-α or its receptor, and FADD (Cleveland and Ihle, 1995); it is absent in the nematode (Ruvkun and Hobert, 1998). This approach was successful; the molecule isolated from *G. cydonium* even comprises two death domains (Wiens *et al.*, 2000*a*). Sequence comparisons revealed that the two domains found in the sponge molecule are to be grouped within the death domain family. It was claimed before that the death domain found in humans comprises relationship to ankyrin motifs (Boldin *et al.*, 1995), an assumption which could be substantiated also experimentally (Müller *et al.*, 2001*a*). Functional assays were performed with allografts from *G. cydonium* which revealed that in rejecting tissue a strong increase of the expression of the death domain-comprising gene (*GCDD2*) occurs (Wiens *et al.*, 2001).

Caspases

In vertebrates, the death domain containing receptors/adapter molecules interact intracellularly with the caspase-8 proenzyme through the death-effector domain with a similar region in the caspase (Grütter, 2000). An adapter-mediated oligomerization causes an activation of the procaspase(s) which undergo cleavage and finally heterodimerization (Cory and Adams, 1998). Finally, upstream caspase(s) activate pro-caspase-3 which in turn is split into the large and small subunits that activate after heterodimerization a factor necessary for the DNase activity to degrade chromatin into the nucleosomal fragments the sign of apoptosis (Fig. 3B).

In Bilateria a series of caspases are involved in the tuned control of apoptosis, starting with caspase-8 in the cascade and ending with caspase-3. Interestingly enough, until now, only one gene has been identified in *G. cydonium* which encodes two transcript forms, both for caspase-8 and for -3 equivalents (Fig. 3A). Two deduced procaspases have been identified, which were termed CAS3l_GEOCY [long form] and CAS3s_GEOCY (short form) (Wiens, to be published). CAS3l_GEOCY can be considered as the procaspase-8 equivalent, due to the presence of the CARD domain (Hofmann *et al.*, 1997). The—probably alternatively spliced product—procaspase-3 equivalent (CAS3s_GEOCY) lacks CARD, but like the CAS3l_GEOCY

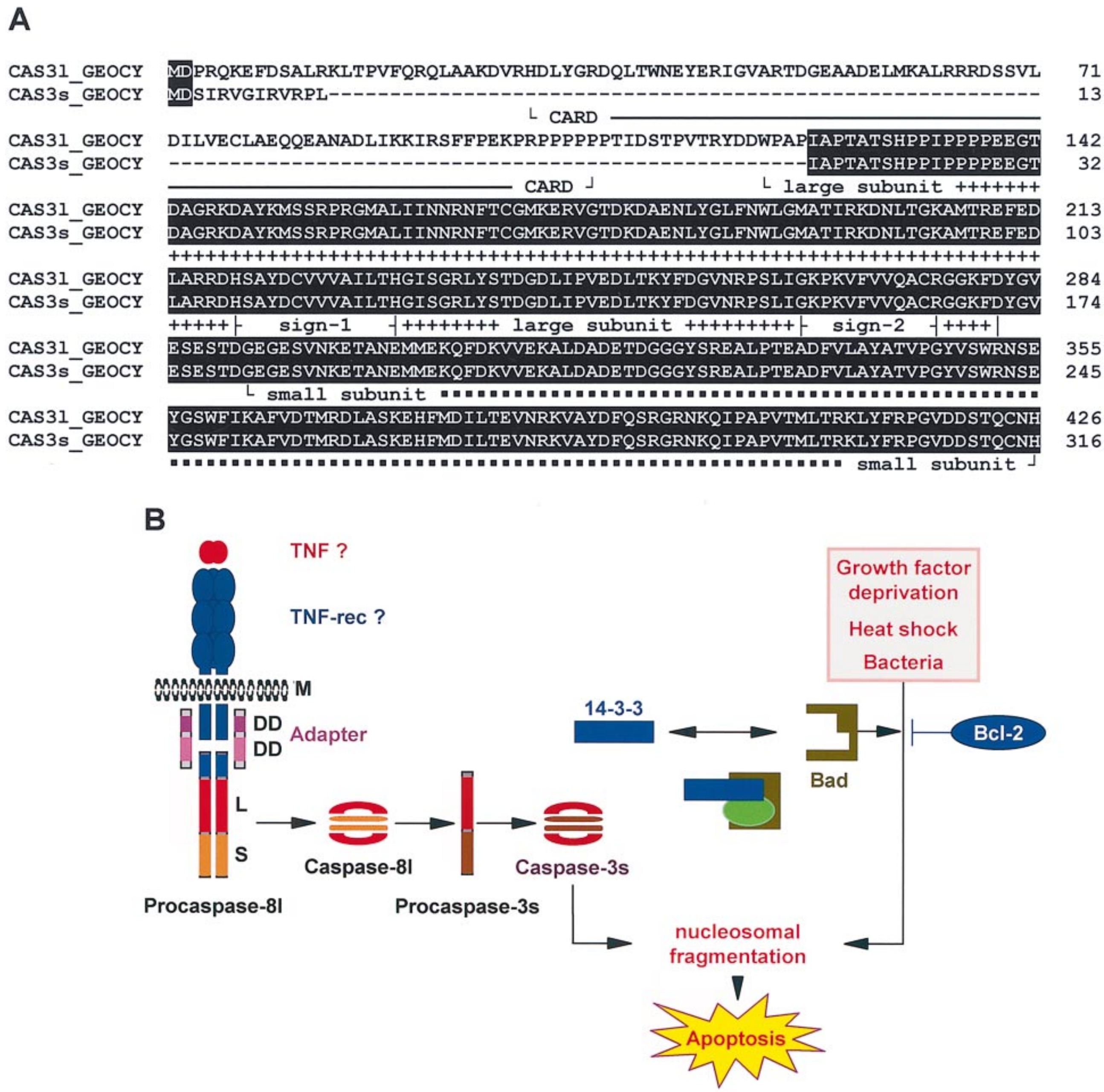

FIG. 3. Apoptotic pathway in sponges. **A.** Alignment of the two *G. cydonium* caspase-3-related polypeptides, deduced from the long (*GEOCYCAS3l*) and short form (*GEOCYCAS3s*) of the corresponding cDNAs. Identical amino acids are in white on black. The borders of the large subunit and of the small subunit (⌊large/small subunit⌋), the CARD segment (⌊CARD⌋) as well as the two signatures (├ sign-1/2 ┤) are marked. **B.** Schematic representation of the known, or suspected members of the apoptotic pathways in sponges. In the extrinsic pathway, the apoptotic signal is initiated by ligand (TNF-like molecule) receptor (TNF-receptor [TNF-rec]) binding which causes the binding of the adapter molecule, the sponge death domain containing molecule. This leads intracellularly to a recruitment of the procaspase-3l (an equivalent to procaspase-8). After activation, followed by heterodimerization of the small (S) and the large subunit (L), this caspase activates the executing procaspase-3s (an equivalent to procaspase-3) which finally causes apoptosis through limited proteolysis and activation of a DNase. In a second pathway other activation processes initiates the cell-death pathway which is under the control of the Bcl-2 and related proteins. If Bcl-2 interacts with Bad apoptosis can proceed; however if Bad becomes trapped by 14–3–3 it undergoes functional inactivation.

sequence, comprises the cleavage sites for the formation of the subunits as well as the two caspase family active sites (Fig. 3A). Functional studies indicate that the two forms of the sponge caspases act in *G. cydonium* in the apoptotic pathway.

Metazoan anti-apoptotic/cell survival proteins

In addition to the activation of the apoptotic process through TNF/TNF-receptor further pathways have been described in Bilateria, which include activation through growth factor deprivation, heat shock or bac-

terial infection (Nicholson and Thornberry, 1997), pathways which have also been described in sponge systems (Wagner *et al.*, 1998). The signal transduction pathway initiated by those factors can be blocked by the function of molecules belonging to the Bcl-2 family (Nicholson and Thornberry, 1997).

In line with the biological evidence that in both *S. domuncula* and in *G. cydonium* apoptosis can be initiated by environmental stress factors, *e.g.*, bacterial load (Wagner *et al.*, 1998) or cadmium (Wagner *et al.*, 1998) an intense screening for members of the Bcl-2 family was started. This effort resulted in the functional analysis of the anti-apoptotic/cell survival proteins from these two sponge species (Wiens *et al.*, 2000*a*, *b*, 2001). The proof that the sponge gene product acts as a cell survival protein was performed by transfection studies using mammalian cells. It could be shown that mammalian cells transfected with the sponge *Bcl-2* related gene confer resistance against heat shock and growth factor deprivation (Wiens *et al.*, 2001).

Besides the molecules of the Bcl-2 family other polypeptides are also known to prevent apoptotic pathways, among them is 14–3–3 molecule which, under ''cytoprotective'' conditions, interacts with the pro-apoptotic Bad after this protein has undergone phosphorylation through Akt. If Bad is bound to 14-3-3 it has lost its binding capacity to the Bcl-2 related molecule Bcl-x_L (Zha *et al.*, 1996). Also this pathway, where 14-3-3 is involved in protection against man-made pollutants, *e.g.*, PCB, has been demonstrated in *G. cydonium* (Wiens *et al.*, 1998); Fig. 3B.

Taken together, the bulk of evidence shows that sponges have a complex apoptotic machinery, which allows the elimination of unwanted tissue (*e.g.*, in allotransplantation) and very likely also in the establishment of an organized body plan.

CONCLUSION: URMETAZOA AS COMPLEX AND INTEGRATED ANIMALS

The question from which organism the Urmetazoa evolved remains open. Frequently, the choanoflagellates have been considered as the sister group of the Metazoa. However, cytological data contradict this view (Karprov and Efremova, 1994), and molecular sequence data from proteins are not available from choanoflagellates. In view of existing data it appears more likely that the Urmetazoa share a common ancestry with the Fungi (Schütze *et al.*, 1999).

An integrated organism, like the Porifera, requires as a prerequisite *cell-cell adhesion systems* which allow the transfer of signals between cell assemblies. The consequence of this level of integration are activation of intracellular signal transduction pathways that result in differential gene expression and cell specialization. One example has been described in primmorphs: only if cell-cell adhesion is allowed the cells undergo cell division and cell differentiation (Müller *et al.*, 1999*b*). In addition, in the primmorph system, morphogen-like molecules, *e.g.*, iron, initiate the complex synthesis of spicule formation (Krasko *et al.*, 2002).

At the next level of integration, the *cell-matrix adhesion* system supports the integration of the functional units of sponges. The major extracellular molecules in sponges are collagen fibrils, which interact with integrin receptors on the cell surface, followed by G-protein and kinase-mediated signal transduction processes (Wimmer *et al.*, 1999).

Cell-cell- and cell-matrix adhesion are the basis for the COLONIAL STAGE of the metazoans and prerequisites for the establishment of integrated systems. These adhesion systems alone are not sufficient for individuation. The stage of individuation can only be reached after the acquisition of an immune system which is paralleled or complemented by a mechanism that eliminates unwanted, which accumulates during development in a multicellular organism; this process is termed apoptosis. Sponges have an amazingly complex *immune system*, which acts against invading microbes or parasites. Furthermore, the immune system is the basis for the individualization; mechanisms have been formed during evolution, which allow for discrimination between self/self and self/non-self.

Apoptosis is the mechanism in Metazoa that guarantees homeostasis. The characteristic apoptotic molecules have been identified in sponges which, together with the functional studies performed, demonstrate that these earliest of metazoans are provided with key regulatory elements for controlled development, tissue homeostasis and defense against pathogens.

Taken together, the phylogenetic oldest, extant metazoan phylum, the Porifera, are provided with complex immune and apoptotic systems that allow the formation of an INTEGRATED SYSTEM (Fig. 1). Considering the fact that the different sponge species are not ''amorphous, asymmetrical creatures'' as suggested (Pechenik, 2000), but comprise a defined phenotype, a sponge might be defined as ''integrated colony'' or an individual, composed of functional units, allowing the formation of a defined body plan.

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