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Expression of autofluorescent proteins reveals a novel protein permeable pathway between cells in the lens core

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SUMMARY

The lens of the eye is composed of concentric layers of tightly packed fiber cells. The oldest fibers, those in the lens core, lose their nuclei and other organelles during terminal differentiation. This is thought to ensure the clarity of the lens. The anucleated core fibers are sustained by gap junction-mediated communication with metabolically active cells near the lens surface. In this study, we expressed autofluorescent proteins and microinjected fluorescent markers to probe cell-to-cell communication in different regions of the developing lens. Our data indicate that a novel cell-cell diffusion pathway becomes patent in the lens core during development. This pathway is remarkable in that it is permeable to proteins and other large molecules and is thus distinct from gap junctions. Diffusion of large molecules probably occurs through regions of membrane fusion observed between neighboring cells in the lens core. Further direct evidence for a continuous plasma membrane system was provided by the observation that exogenous membrane proteins expressed in one core fiber cell were able to diffuse laterally into the membranes of adjacent fibers. Thus, the lens core appears to represent a true syncytium within which both membrane proteins and cytoplasmic proteins freely diffuse. Significantly, the outermost edge of the core syncytium encompasses a shell of nucleated, transcriptionally-competent, fiber cells. This arrangement could facilitate the delivery of newly synthesized protein components to the aged and metabolically quiescent cells in the center of the lens.

Key words: Green fluorescent protein, Two-photon microscopy, Lens, Differentiation, Communication, Cell fusion

INTRODUCTION

The lens grows throughout life by the deposition of newly differentiated fiber cells at its surface. Preexisting fibers are progressively buried. As a normal feature of differentiation, fiber cells degrade their nuclei and other organelles, resulting in the formation of an organelle-free zone in the lens core (Bassnett and Beebe, 1992; Bassnett, 1995). This process guarantees that the optical path is free of light-scattering structures and thereby ensures the transparency of the lens. The mature lens is thus composed of a layer of young, metabolically-active, surface fiber cells overlying a core of relatively inactive, older fibers.

Communication between cells near the surface and those in the lens core is critical for normal function (Goodenough et al., 1980). Metabolites enter the surface cells and diffuse from cell to cell, via gap junctions, into the lens core. However, whereas small molecules are free to diffuse in this fashion, the proteinaceous components of fiber cells are not. Being too large to pass through gap junctions, proteins are trapped within the cells in which they were originally synthesized.

Protein synthesis is thought to cease shortly after the nuclei and other organelles are degraded. This occurs as early as embryonic day 12 (E12), in the chicken lens (Bassnett and Beebe, 1992). As there is no cell turnover in the core of the lens, core fibers and their macromolecular contents must persist for the life of the individual. In the absence of new synthesis, lens proteins are exposed to a lifetime of potentially damaging environmental stress. Indeed, the accumulation of damage to aging proteins in the lens core is thought to result in cataract formation, the most common cause of blindness in the world (Young, 1991).

Advances in gene delivery technology and confocal microscopy have recently allowed the expression and fate of exogenous proteins to be visualized in intact, living lenses (Shestopalov and Bassnett, 1999, 2000). In the present work, we used this combined approach to examine the properties of a novel protein-permeable pathway that forms between fiber cells in the core of the developing lens. The results are discussed in relation to a new model of lens metabolism.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chicken eggs were incubated at 38°C until use.

Vectors

This study utilized a variety of plasmid vectors encoding enhanced green fluorescent protein (EGFP) and two of its spectral variants, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP). pEYFP-Nuc (Clontech) encodes a protein in which three tandem repeats of the simian virus T-antigen nuclear localization signal (Lanford et al., 1986) are fused to the C terminus of EYFP. pEYFP-Mem (Clontech) encodes a fusion protein in which the N-terminal 20 amino acids of neuromodulin are fused to the N terminus of EYFP. This fusion protein is primarily targeted to the inner face of the plasma membrane and can, therefore, be used to visualize plasma membrane dynamics directly (Moriyoshi et al., 1996). pCMV-ECFP was generated by cloning an ECFP-containing *Bam*HI-*Not*I fragment from the promoterless pECFP plasmid (Clontech) into pEGFP-N1 (Clontech). In the resulting plasmid, ECFP replaces EGFP and its expression is driven by the CMV_{IE} promoter. We have shown previously that the hybrid CAG promoter described by Miyazaki et al. (1989) is extremely potent in the lens (Shestopalov and Bassnett, 2000). This promoter was incorporated into pCAG-EGFP, pCAG-CD46 and pCAG-MIP/EGFP. CD46 is a widely expressed human membrane protein (Lublin et al., 1988) which we have used previously to probe patterns of plasma membrane synthesis in the embryonic chicken lens (Shestopalov and Bassnett, 1999). MIP (or aquaporin 0) is the major intrinsic protein of the vertebrate lens. pCAG-MIP/EGFP was generated by cloning a 965 bp cDNA, encompassing the complete coding sequence of wild-type mouse MIP (Shiels and Bassnett, 1996), into pCAG-EGFP. The resulting plasmid encodes a fusion protein in which EGFP is fused to the C terminus of MIP.

Vesicle injection

Injections were made into the lens vesicle on E3.5 as described (Jiang and Goodenough, 1998; Shestopalov and Bassnett, 2000). Injections into the lumen of the vesicle resulted in EGFP expression in a few, centrally located fibers. Eccentric injections into the vesicle wall resulted in labeling of core and peripheral fiber cells.

Microinjection of plasmids into individual fiber cells

The pCAG-EGFP plasmid was pressure microinjected into individual fiber cells of organ cultured lenses. Injections were made in or near the fiber cell nuclei as described (Shestopalov and Bassnett, 1999). EGFP expression was visualized 20 hours after injection.

Microinjection of fluorescent dyes

Fiber cells were injected with a cocktail of 1 mM carboxyfluorescein (CF) and 100 mg/ml 40 kDa tetramethylrhodamine-dextran (TD; Molecular Probes) in water. The TD was cleaned using a 10 kDa molecular mass cutoff Microcentricon filter (Millipore) to remove low molecular mass components (Fraser, 1996). Dyes were introduced into the tip of the injection pipette and overlaid with 0.6 M LiCl. Dye was injected iontophoretically for 30 seconds at 0.5 μA using a dual current generator (Model 260, World Precision Instruments, Inc., Sarasota, Fl). During injection lenses were immobilized in 1.5% low melting point agarose dissolved in artificial aqueous humor (AAH; for composition see Bassnett et al., 1990). Injected dyes were visualized using long-working distance water immersion objectives. The diffusion of injected dyes was visualized by time lapse imaging using a cooled CCD camera (Sensys, Photometrics).

Immunocytochemistry

CD46 expression was detected by immunocytochemistry with TRA2-10 antibody as described (Shestopalov and Bassnett, 1999). The distribution of the primary antibody was visualized using anti-mouse IgG second antibody conjugated to the Alexa 568 fluorochrome (Molecular Probes, Eugene, OR).

Western blot

Lenses were microdissected into core or peripheral regions. Samples were separated on 10% SDS-PAGE mini-gels and transferred to nitrocellulose. Membranes were incubated with polyclonal anti-GFP (Molecular Probes) and bound antibody was detected by ECL (Amersham).

Confocal microscopy, two-photon microscopy and volume rendering

For confocal microscopy, intact lenses or fixed lens slices were visualized using a Zeiss 410 LSM microscope equipped with an argon krypton laser. Although conventional confocal microscopes provide high resolution images near the surface of thick specimens, image quality rapidly deteriorates in the deeper tissue layers. Therefore we used two-photon excitation microscopy to visualize the three-dimensional organization of fiber cells in the embryonic lens. Two photon excitation arises from the near simultaneous absorption of two long wavelength photons by a fluorochrome (for a review see Piston, 1999). Only at the focal point of the objective lens are the photons at high enough density to cause appreciable fluorescence. Thus, two photon microscopes are especially well suited to viewing thick specimens because out-of-focus haze and bleaching are substantially reduced. The two photon microscope used here was based on a Bio-Rad MRC1024 laser scanning microscope. The microscope was equipped with a mode locked, tunable Ti-sapphire laser. To visualize the fiber cell membrane architecture in three dimensions, 200 μm equatorial slices were prepared from fixed lenses and stained with the fluorescent lipophilic dye $DiOC_6$ as described (Bassnett, 1995). Fluorescence was produced by two-photon excitation at 890 nm. Images were collected at 0.5 μm intervals through 100-200 μm of tissue depth using a 1.2 NA ×60 water immersion objective (Olympus).

The image stack (typically comprised of 200-300 optical sections) was reconstructed using VoxBlast software (Vaytek, Inc., Fairfield, IA, USA). To localize regions of fusion between neighboring fiber cells, a seeding algorithm was employed. A voxel seed was placed in the cytoplasm of individual fibers in the reconstructed tissue volume and allowed to flood fill the cytoplasm. If the filled cell was bounded by an uninterrupted plasma membrane (i.e. the cell was not fused to another) the flood fill was retained within the seeded cell. However, if the seeded cell was fused to one or more neighbors the flood fill could escape through the interrupted membrane. By seeding each cell in turn it was possible to visualize the labyrinthine connections within the reconstructed tissue volume.

RESULTS

We injected an enhanced green fluorescent protein (EGFP) expression plasmid (pCAG-EGFP) into the lumen of the chicken lens vesicle at E3.5. Following injection, embryos were returned to the incubator and allowed to develop. At E7, EGFP was detected in the cytoplasm of a few primary fiber cells located in the lens core (Fig. 1a). By E12, EGFP was distributed throughout the core in a diffuse sphere of fluorescence that encompassed the cytoplasm of thousands of cells (Fig. 1b). We hypothesized that the sphere of fluorescence resulted from the diffusion of EGFP from a few fiber cells in which it was originally synthesized into the cytoplasm of many neighboring fibers. This implies that the lens core is a functional syncytium. However, on the basis of the original observations we could not exclude the possibility that each cell within the fluorescent sphere synthesized EGFP independently from the pCAG-EGFP template. To resolve this issue, we devised a strategy for identifying those cells in which EGFP originated. For this purpose, two plasmids encoding spectral variants of EGFP were used. Plasmids encoding enhanced cyan

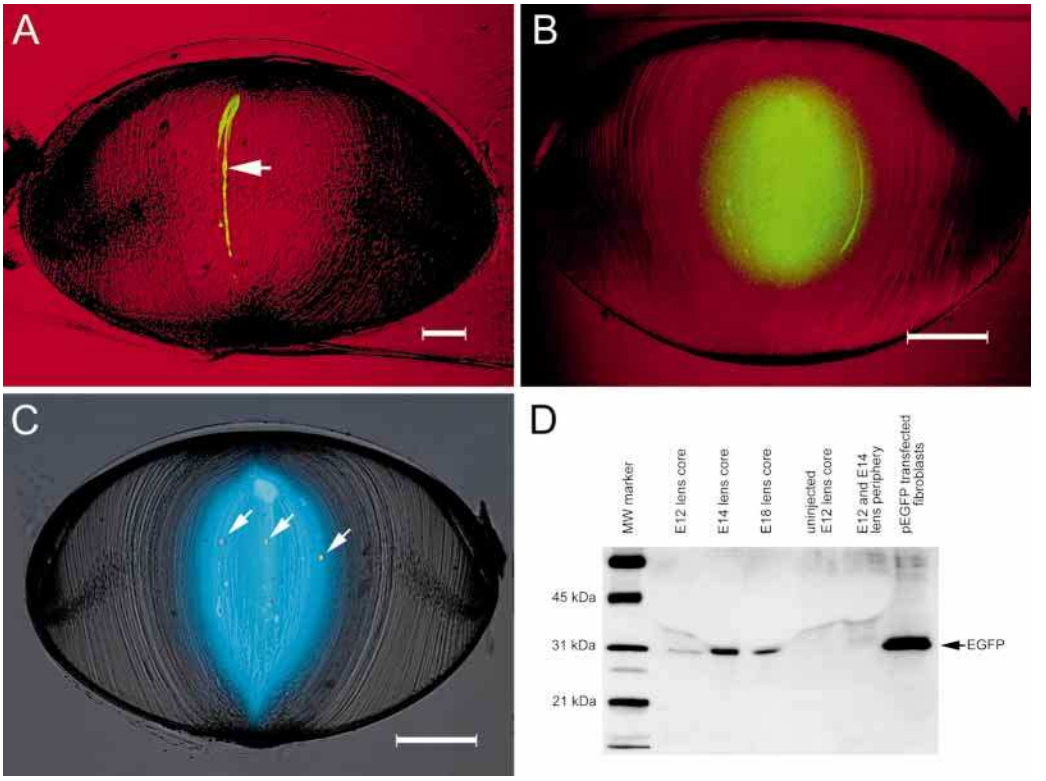

Fig. 1. Cell-to-cell diffusion of fluorescent proteins in the lens core. (A) Injection of pCAG-EGFP into the lumen of the lens vesicle results, by E7, in EGFP expression in a few primary fiber cells (arrow). (B) At E12, EGFP fluorescence is observed throughout the lens core. (C) E11 lenses co-injected with pCMV-ECFP and pEYFP-nuc contain a few, centrally located fiber cells with yellow nuclei (arrows) surrounded by a diffuse cloud of ECFP (blue) which encompasses thousands of additional fiber cells. (D) Western blot of material micro-dissected from various regions of pCAG-EGFP-injected or control lenses. Note the absence of proteolytic breakdown products. Bars: (A) 100 μm; (B and C) 250 μm.

fluorescent protein (ECFP) and a nuclear targeted enhanced yellow fluorescent protein (EYFP-Nuc) were mixed together and injected into the lens vesicle. By E8, several labeled cells (usually 10-20) were observed in the lens core. Of these, >80% expressed both ECFP and EYFP-Nuc (data not shown). At E11, a few cells near the center of the lens contained fluorescent yellow nuclei due to the presence of EYFP-Nuc (Fig. 1c). In contrast, diffuse ECFP fluorescence was present in the cytoplasm of hundreds of central fiber cells. If competent fiber cells expressed both plasmids then ECFP originated in cells with yellow nuclei. These data demonstrated that ECFP was synthesized by a few central cells and diffused subsequently into many others. The border of the resulting fluorescent sphere defined the size and shape of the core syncytium.

If proteolysis of EGFP occurred in the cytoplasm of expressing cells then this could provide an alternative explanation for cell-to-cell diffusion of the protein. Fragments of EGFP might be sufficiently small (i.e. <1.5 kDa) to diffuse via the gap junctions that join fiber cells (Goodenough, 1992).

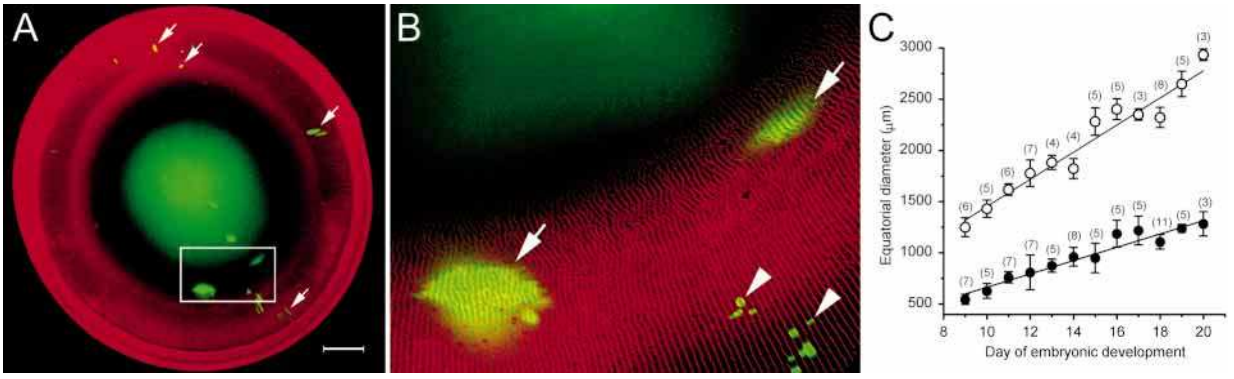

Fig. 2. The core syncytium increases in volume during embryonic development. (A) Equatorial slice of an E17 lens in which pCAG-EGFP was injected eccentrically into the lens vesicle. Note the sphere of EGFP fluorescence (green) in the center of the lens and scattered EGFP-expressing fiber cells in the periphery (arrows). The lens was pre-incubated in Texas Red dextran (red) prior to fixation and slicing to visualize the tissue architecture. (B) Higher magnification view of the boxed region from (A). Note the diffusion of EGFP from expressing fiber cells in the deep cell layers (arrows) and the single cell labeling in the peripheral layers (arrowheads). (C) Growth of the lens (open circles) and the sphere of EGFP fluorescence (filled circles) during embryonic development. The diameters of the lens and the sphere of EGFP fluorescence were computed directly from digital images. The position of the boundary of the fluorescent sphere was judged by eye. Data represent the mean ± S.D. of (n) lenses. Scale bar: (A) 250 μm.

However, because the fluorescence properties of EGFP depend on its tertiary structure, it is unlikely that fragments of the parent molecule would be fluorescent. In vitro mutagenesis experiments have demonstrated that no more than 15 terminal amino acids can be deleted before the fluorescent properties of wild-type GFP are lost (Li et al., 1997). To confirm that proteolysis of EGFP did not occur in lens fibers in vivo, we performed western blot analysis on material micro-dissected from various regions of pCAG-EGFP vesicle-injected lenses (Fig. 1d). There was no indication of proteolytic cleavage of EGFP at any stage examined.

Eccentric injection of pCAG-EGFP into the wall of the lens vesicle resulted in EGFP expression in peripheral and core fibers. At late developmental stages (>E15), such lenses exhibited a complex pattern of fluorescence (Fig. 2a,b). The core of these lenses was filled with a diffuse sphere of EGFP fluorescence; presumably resulting from prior diffusion of EGFP from a few centrally located fiber cells. In the deep cortex of the lens, EGFP was beginning to diffuse from expressing cells. In contrast, near the surface of the lens, EGFP-fluorescence was restricted to the cytoplasm of individual fiber cells. These data suggest that the core syncytium expands during development and eventually encompasses secondary fiber cells that originally lay beyond its border.

Interestingly, in the lens core, we noted a reciprocal distribution of EGFP and the Texas Red Dextran (TRD) counterstain. EGFP is an intracellular protein whereas TRD labels the extracellular space. At earlier developmental stages, TRD readily permeated the extracellular space between core fibers (data not shown). The establishment of the core syncytium thus appears to coincide temporally and spatially with the obliteration of extracellular space in the lens core.

The rate of expansion of the core syncytium was determined by measuring the equatorial diameter of the fluorescent sphere on consecutive days of development (Fig. 2c). The sphere was first apparent on E9 and increased linearly in diameter at a rate of 65 μm (approximately 20 cell layers) per day. This was approximately half the growth rate of the lens as a whole during this period (132 μm/day).

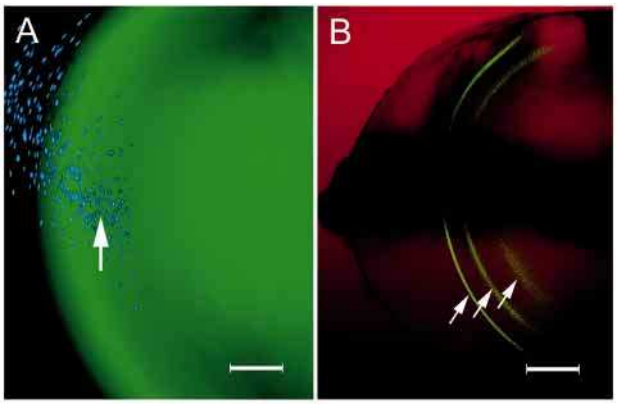

Fig. 3. The core syncytium contains a layer of nucleated, transcriptionally-active fiber cells. (A) Midsagittal slice of a pCAG-EGFP vesicle-injected E17 lens stained with DAPI (blue). The DAPI stained nuclei extend approximately 150 μm into the sphere of EGFP fluorescence (green). The position of the last intact nucleus is indicated (arrow). (B) The pCAG-EGFP expression plasmid was injected directly into the cytoplasm of three cells (arrows) at different depths in an organ cultured E15 lens. Injections into nucleated fiber cells within the core syncytium resulted in a diffuse pattern of fluorescence encompassing many fibers. Injections into more peripheral fibers resulted in single cell EGFP labeling. Bars: (A) 100 μm; (B) 200 μm.

During development, core fiber cells degrade their nuclei (Bassnett and Mataic, 1997) and are thereby transcriptionally silenced (Shestopalov and Bassnett, 1999). We sought to determine whether the denucleation process occurs before or after cells are incorporated into the expanding syncytium. By staining vesicle-injected lenses with the DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), we co-visualized the distribution of fiber cell nuclei and the border of the syncytium (Fig. 3a). These data demonstrated that the core syncytium contained a shell of nucleated fiber cells approximately 150 μm thick. To test whether these cells were transcriptionally and translationally competent, we

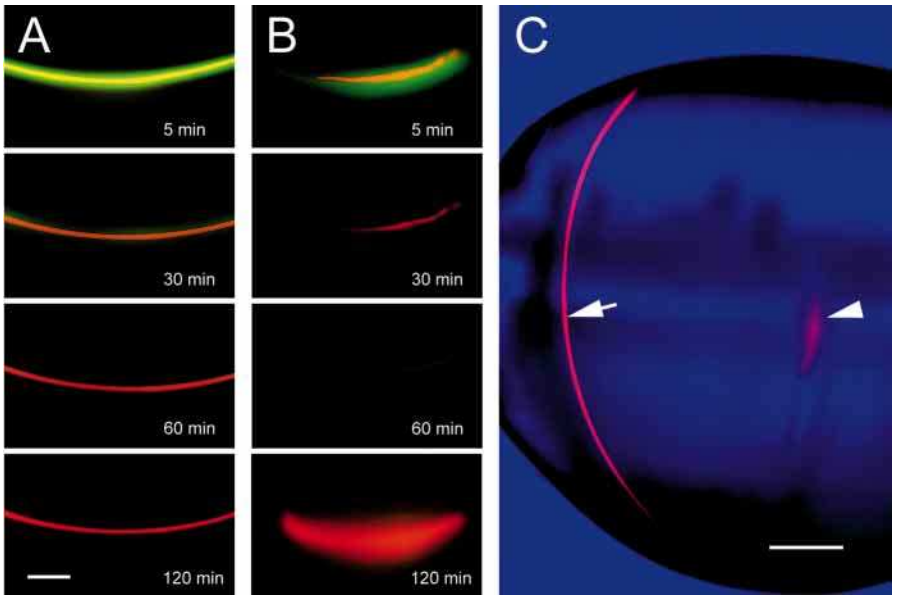

Fig. 4. Co-injection of carboxyfluorescein (CF) and 40 kDa tetramethylrhodamine dextran (TD) demonstrates the existence of a syncytium in the lens core. (A) A cocktail of CF (green) and TMR-dextran (red) was injected into a peripheral fiber cell of an E17 lens. After 120 minutes CF had diffused from the injected cell but TD was retained. (B) A similar injection was made into a fiber cell located in the lens core. In this case, both CF and TD diffused from the injected cell. After 120 minutes, the TD fluorescence was so weak in the injected cell that the exposure time on the CCD camera had to be increased 10-fold to visualize it. With the new camera settings, TD fluorescence was observed in the injected cell and neighboring cells. (C) Low magnification image of an E17 lens 3 hours after TD injections into peripheral (arrow) and core (arrowhead) fiber cells. Note the diffusion of TD from the core fiber cell and its retention in the peripheral cell. Bars: (A and B) 100 μm; (C) 250 μm.

microinjected pCAG-EGFP into fiber cells bordering the syncytium (Fig. 3b). Peripheral cells showed no evidence of EGFP diffusion. However, injections into the deeper cell layers resulted in diffusion of EGFP from the expressing cell into the cytoplasm of many neighbors demonstrating that the core syncytium contains transcriptionally competent cells.

To study the dynamics of cell-cell communication in the lens, we visualized the diffusion of carboxyfluorescein (CF; 376 Da) and tetramethylrhodamine-dextran (TD; 40 kDa) injected iontophoretically into various regions of the lens (Fig. 4). By injecting a cocktail of both molecules, gap junction-dependent and -independent pathways were distinguished. In the periphery of the lens, CF diffused quickly from the injected fiber into neighboring cells and, after 2 hours, the CF signal was no longer detectable in the injected cell (Fig. 4a). In contrast, TD was retained in the injected cell. Even after 2 hours, there was no evidence of diffusion into the cytoplasm of neighboring cells, and the TD fluorescence intensity in the injected cell was indistinguishable from that measured immediately after injection. These observations support the hypothesis that peripheral fiber cells are joined solely by gap junctions, and that these are impermeable to large molecules. Injection of the dye cocktail into core fibers (Fig. 4b) resulted in a different pattern of diffusion to that observed in the periphery. In the lens core, CF diffused rapidly from the injected cell. Likewise, TD diffused from the injected cell into the cytoplasm of neighboring cells (albeit more slowly than CF). Lower magnification images (Fig. 4c) revealed the fate of TD 3 hours after injection into peripheral or core fiber cells. In the periphery, the TD fluorescence was retained in the injected cell. In the core, the fluorescence was distributed as a diffuse cloud of cytoplasmic fluorescence centered about the injected cell, but encompassing hundreds of uninjected cells. These observations suggest that, in the lens core, cells are connected via a pathway permeable to molecules of at least 40 kDa.

To probe plasma membrane organization in different regions of the lens, we examined the distribution of three exogenous membrane proteins (CD46, MIP-EGFP and EYPF-Mem) introduced into the lens by injection of appropriate plasmid vectors into the lens vesicle at E3.5.

CD46 is a well characterized membrane glycoprotein that is widely expressed in human tissues where it plays a critical role in inhibiting complement activation on host cells (Liszewski et al., 1996). A basolateral targeting sequence is present at the C terminus of CD46 (Maisner et al., 1997) and in the chicken lens the protein is properly targeted to the lateral membrane of lens fiber cells (Shestopalov and Bassnett, 1999). We have previously used the expression of human CD46 to probe membrane synthesis in the developing avian lens. In these studies, the pCAG-CD46 plasmid was mixed with pEYFP-Nuc to enable cells originally expressing CD46 to be identified. In peripheral fiber cells at E10, the CD46 immunofluorescence was restricted to the plasma membranes of individual expressing cells (Fig. 5a). Expressing cells were identified by the presence of a fluorescent yellow nucleus in addition to CD46 immunofluorescence. However, cross sections of CD46-expressing cells in the lens core at this age revealed a different pattern; CD46 was present in the membrane of expressing cells (identified by their yellow nuclei) and the membranes of neighboring, non-expressing cells (Fig. 5b). At later stages (>E12), all nuclei are degraded in core fiber cells (Bassnett and Beebe, 1992). Consequently, EYFP-Nuc fluorescence was absent. However, CD46 fluorescence persisted in the membranes of fiber cells in the lens core (Fig. 5c). The clusters of CD46-positive fiber cells were generally larger than at earlier stages, suggesting the continued diffusion of CD46. These data suggest that during differentiation CD46 diffuses from the membrane of the cell in which it was originally synthesized and spreads into the membranes of surrounding fiber cells.

Although we have previously used the expression of human CD46 in the chicken lens to visualize patterns of membrane synthesis (Shestopalov and Bassnett, 1999), little information is available regarding the trafficking or behavior of this protein in the lens. To ensure that the observed lateral intercellular diffusion of CD46 was not an artifact associated with this

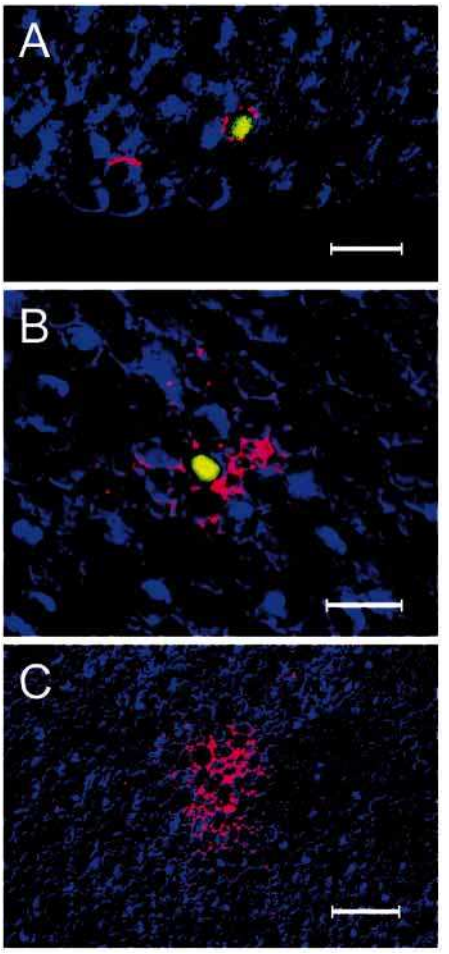

Fig. 5. Lateral diffusion of membrane proteins in different regions of the lens. Lenses were vesicle-injected with a mixture of pEYFP-Nuc and pCAG-CD46. Expressing cells were identified by the presence of a yellow nucleus (due to the accumulation of EYFP-Nuc) and/or CD46 immunofluorescence (red). (A) At E10, peripheral fiber cells containing a yellow nucleus and CD46 plasma membrane staining were present. CD46 immunofluorescence was restricted to the plasma membrane of the expressing cell. (B) In contrast, in the core region of E10 lenses, CD46 was present in the plasma membrane of expressing cells and in the membranes of a cluster of surrounding, non-expressing cells. (C) At E14, fiber cells in the lens core have lost their nuclei; however, CD46 immunofluorescence persisted in the plasma membranes of a cluster of central cells. Scale bars: (A) 15 μm; (B) 10 μm; (C) 25 μm.

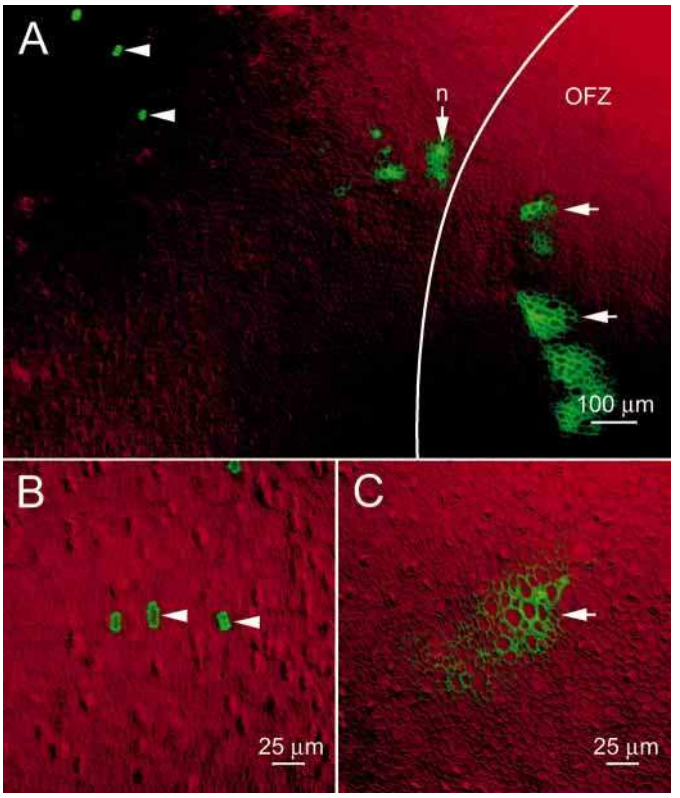

Fig. 6. Diffusion of MIP-EGFP fusion protein during fiber cell differentiation at E12. (A) Eccentric injection of a cocktail of pMIP-EGFP and pEYFP-Nuc into the lens vesicle resulted in expression of MIP-EGFP and EYFP-Nuc in scattered cells throughout the lens at E12. In the lens core, MIP-EGFP was detected in the membranes of clusters of cells (arrows). At the border of the organelle free zone (OFZ), where nucleated fibers are still present, MIP-EGFP fluorescence was present in the membranes of several fiber cells centered about a fiber in which the cell nucleus (n) was labeled by EYFP-Nuc. In the lens periphery, MIP-EGFP fluorescence was restricted to the membranes of individual expressing cells (arrowheads). Higher magnification images of cells in the cortex and core of the lens are shown in B and C, respectively. Note that, due to spectral overlap between fluorochromes, it was not possible to distinguish between MIP-EGFP and EYFP-Nuc fluorescence (consequently, both are shown in green in this figure). However, in independent experiments, we verified that the spatial distribution of the two probes did not overlap: EYFP-Nuc always localized to fiber cell nuclei and MIP-EGFP was always restricted to the plasma membrane (data not shown). Thus, we were able to unambiguously identify the nucleus arrowed in A.

particular protein, we expressed an MIP-EGFP fusion protein in the lens. MIP is the most abundant integral membrane protein in the lens, accounting for more than 50% of the plasma membrane protein (Broekhuyse et al., 1976). We reasoned that an MIP fusion protein was likely to behave in a physiological manner. Following vesicle injection of pCAG-MIP/EGFP, plasma membrane fluorescence was observed (Fig. 6). In superficial fibers at E12, the MIP-EGFP fusion protein was restricted to the plasma membranes of individual expressing cells (Fig. 6a,b). In the deeper cell layers, however, MIP/EGFP was detected in the plasma membranes of clusters of 50 or more neighboring fiber cells. Similar data were also obtained by expressing a third membrane protein, EYFP-Mem, in lens fiber cells (data not shown). The redistribution of these three membrane proteins in the lens core suggests that, as differentiation proceeds, the membranes of adjacent fiber cells become joined in some fashion, facilitating the exchange of membrane components via lateral diffusion in the plane of a continuous membrane.

Plausible conduits for the intercellular diffusion of cytoplasmic and membrane components are regions of fusion between adjacent fiber cells. Such fusions have been identified in the adult lenses of several species (Kuszak, 1995; Kuszak at al., 1985, 1989) but have not been described previously in the embryonic chicken lens. To determine whether membrane fusions are present in the center of the chicken lens, equatorial slices were incubated with a fluorescent lipophilic dye to visualize plasma membrane structure (Bassnett, 1995). Slices were imaged by two photon microscopy. Stacks of optical sections were collected from regions of the lens core and superficial fiber layers. The image stacks were reconstructed using volume rendering software and examined for the presence of cell fusions using a voxel seeding algorithm. In this fashion, the labyrinthine connections between fiber cells were mapped in three dimensions. Fig. 7 shows three-dimensional reconstructions/fusion distribution maps for reconstructed volumes from the lens cortex (Fig. 7A,C) and core (Fig. 7B,D). Fused cells were not observed in the periphery but were relatively common in the lens core. The morphology of the fused region was visualized in detail by selecting appropriate alpha values in the rendering program thereby controlling the relative opacity of the flood filled volume and surrounding cells. An example of a pair of fused fiber cells visualized in this way is shown in Fig. 8. In this example, the fused region extended for almost 30 µm. The reconstructions covered approximately one third (150 µm) of the length of the core fiber cells. Within the reconstructed tissue volume ~10% of the fiber cells were fused to their neighbors. If fusions were distributed evenly along the cell, this would suggest that approximately 30% of core fibers were fused.

DISCUSSION

During development a syncytium is established in the lens core. Plausible conduits for diffusion of large molecules are cell-to-cell fusions, which are a prominent feature of most, perhaps all, lenses (Kuszak, 1995; Kuszak at al., 1985, 1989). Fusions have been proposed to function in the establishment of lens architecture (by providing nodal points where columns of fiber cells bifurcate; Kuszak et al., 1989) but the present data suggest additional physiological or metabolic roles. The hypothesis that fusions constitute the diffusion pathway for large molecules is supported by the observation that fusions were observed in the lens core (where proteins freely diffuse) but were not observed in the periphery (where proteins do not diffuse between cells).

Membrane protein diffusion is thought to occur only in the plane of a continuous membrane. Therefore, cell-to-cell diffusion of CD46, EYFP-Mem or MIP-EGFP in the lens core, is further evidence for the presence of fusions between cells in this region. Because we never observed an instance in which these membrane proteins failed to diffuse between neighboring

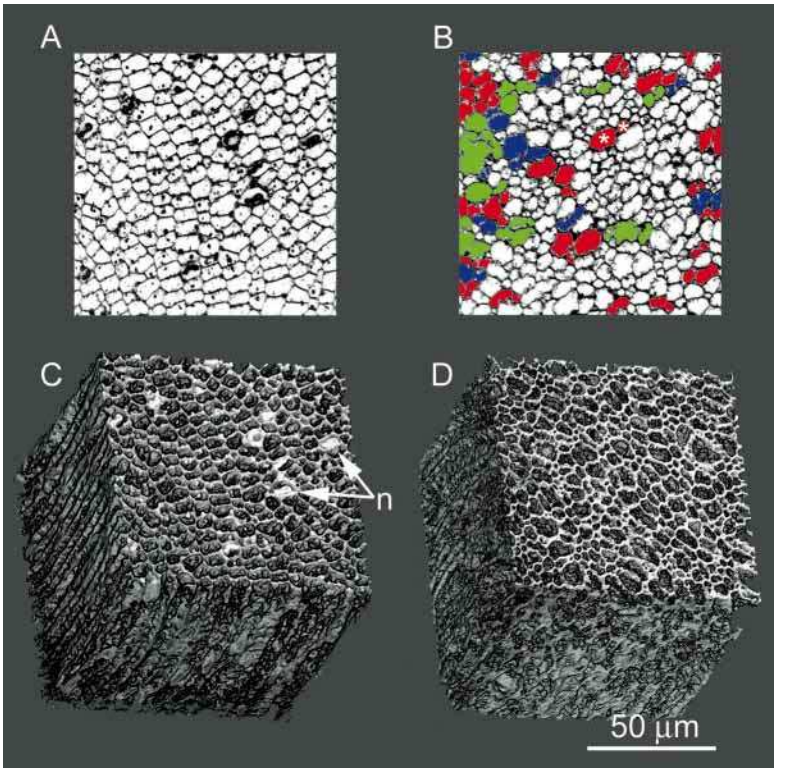

Fig. 7. Three-dimensional reconstruction and mapping of cell fusions in an E17 lens. Equatorial lens slices were stained with $DiOC_6$ to visualize the plasma membranes of the fiber cells and stacks of optical sections were collected from the cortical (A,C) or core regions (B,D) of the slice using a two photon microscope. A voxel seeding algorithm was used to detect regions of cell fusion within the reconstructed volume. The cortical reconstructions (A,C) featured regularly packed cells containing nuclei (n) and other organelles (D). Voxel seeding failed to detect fusions between cortical fiber cells. In contrast, reconstructions of core fibers revealed irregularly packed cells of variable cross sectional area. Voxel seeding revealed that many of these cells were fused. Fused cells are identified by color coding in the two-dimensional image shown in B. Identically colored regions represent regions of fusion between two or more cells. The pair of cells marked by * were used for the reconstruction shown in Fig. 8.

cell membranes, we hypothesize that all core fibers are fused at some point along their length for at least some period of time. A true syncytium would require that fiber cells fuse with at least two (and probably more) neighbors but inspection of reconstructed core fibers revealed fusions to be numerous but not ubiquitous. However, it is likely that the number of fusions observed by two photon microscopy represented an underestimate. Because of the limited resolution of the microscope (especially in the z-axis) and the highly folded nature of the fiber membranes, only relatively large fusions (>5 μm in diameter) would be detected. Similarly, if fusions were transient phenomena they would be underestimated. Although fusions are the most likely pathway for the diffusion of large molecules, the existence of other mechanisms cannot be excluded.

The fusion of progenitor cells into multinucleated syncytia is a characteristic of several tissues. In the placenta, cytotrophoblast cells fuse to form the syncytiotrophoblast. Macrophages fuse to form multinucleated giant cells during inflammatory reactions and osteoclasts and myotubes form from the fusion of monocytes and myoblasts respectively. A difference between these examples and cell fusion in the lens core is that fiber cell fusion appears to involve only a small fraction of the surface of a given cell. The remainder of the cell retains its characteristic cross sectional profile bounded by a distinct membrane. Thus, cell fusions in the lens do not grossly disturb the packing arrangement of the cells or the highly ordered tissue architecture.

Thermodynamic considerations suggest that, once formed, fusion pores between fibers should quickly expand. This evidently does not occur. The mechanism by which fusion pores are stabilized and constrained in diameter is unknown, although the fiber cytoskeleton is likely to play a role.

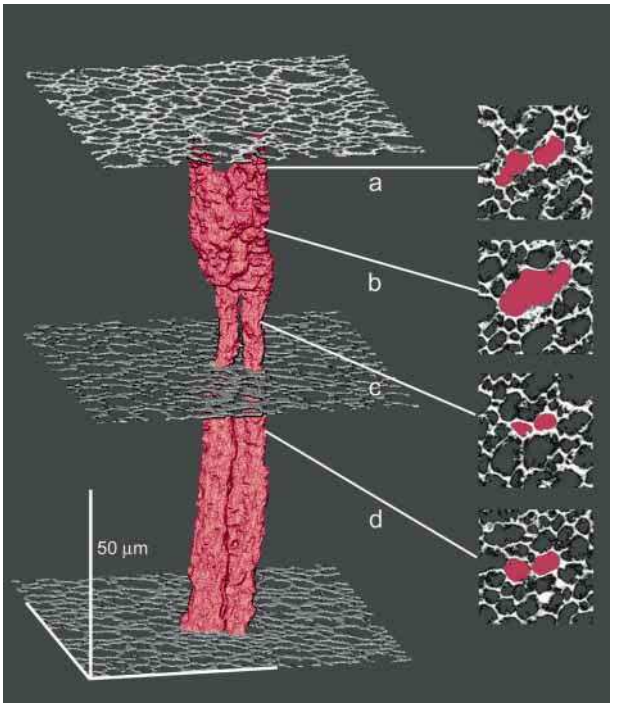

Fig. 8. Three-dimensional reconstruction of the cytoplasm of two neighboring fiber cells in the lens core. The final image was reconstructed from 269 individual optical sections collected at 0.5 μm z-axis intervals. Selected optical sections (a-d) are shown. The reconstructed fibers are present as discrete cells throughout much of their length (a, c and d). However, the septum of plasma membrane that separates the cytoplasm of the cells is absent for approximately 30 μm. In this region, the cytoplasmic contents of the two fibers are free to mingle (b).

The functional implications of a syncytial organization in the lens core are considerable. Optically, the establishment of cell fusions may reduce light scatter at cell boundaries, especially as the formation of the syncytium coincides with the obliteration of the extracellular space.

To focus on objects at different distances, the lenses of many species (including chickens and humans) are able to change shape (accommodate). In these lenses, cytoplasmic continuity between cells may allow for redistribution of hydrostatic pressure generated during accommodation.

Studies using labeled metabolites have demonstrated the importance of an intercellular pathway in the delivery of nutrients from the ocular fluids to cells in the core (Goodenough et al., 1980). In the periphery of the lens, this pathway is believed to consist entirely of gap junctions. However, the connexin proteins of which gap junctions are composed are usually labile, with half-lives of hours or days (Berthoud et al., 1999). As early as E12, cells in the lens core lose the ability to incorporate new membrane components (Bassnett, 1995). If gap junctions were the only means of communication in this region then they would have to remain functional for the life of the individual. The syncytium described here may represent a stable, long-lived alternative, allowing intercellular communication to persist in the lens core, even in the absence of protein synthesis. Our data, thus, suggest a model in which gap junction-mediated communication in the peripheral cells is in series with fusion-mediated coupling in the core.

Unlike gap junctions, cell fusions are not expected to function as gated channels. One prediction of the model, therefore, would be that treatments (such as intracellular acidification) that reduce gap junction permeability should uncouple surface cells but have little effect on cell coupling in the lens core. In fact, perfusion with CO_2 has been shown to specifically uncouple the peripheral fibers without effecting the coupling of core fibers (Mathias et al., 1991). These results, however, are also consistent with the notion that post-translational modification of connexin proteins in the core renders them insensitive to pH (Lin et al., 1998). In humans, two forms of lens opacification are distinguished; cortical and nuclear. Cortical opacities are usually localized, involving single cells or bundles of cells. The discrete nature of cortical opacities suggests that the damaged fibers uncouple from their neighbors. In contrast, nuclear opacities are usually characterized by the homogeneous opacification of the entire core region. These clinical observations are consistent with a model in which the connections between peripheral fiber cells can be modulated but those between core fibers cannot.

The observation that the core syncytium encompasses a layer of nucleated, transcriptionally-active fibers suggests a further metabolic role. In principle, newly-synthesized proteins could diffuse from peripheral cells into the center of the lens and aged or partially-denatured proteins could move in the opposite direction. The diffusion coefficient of proteins in the fiber cytoplasm would likely be small and, depending on the relative distribution of cell fusions, the pathway could be tortuous. Furthermore, as the lens grows, the distance between anucleated fiber cells in the core and nucleated fibers at the periphery increases. Nevertheless, the syncytium could facilitate the slow exchange of cytoplasmic proteins between neighboring fiber cells in the core and between core fibers and superficial fibers. One implication of this model is that cytoplasmic proteins in core fibers could be considerably younger than the cells themselves. Metabolic labeling strategies could be used to test this prediction directly.

Lens transparency requires that cells in the light path are free of light-scattering structures (organelles) yet maintain their integrity over the life of the organism. The syncytial organization described here may represent a solution to these seemingly paradoxical demands.

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