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Insertion of MP20 into lens fibre cell plasma membranes correlates with the formation of an extracellular diffusion barrier

Angus C. Grey[a], Marc D. Jacobs[a], Tamir Gonen[b], Joerg Kistler[b], Paul J. Donaldson[a,*]

[a]*Department of Physiology, School of Medical Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*
[b]*School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*

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Abstract

It is known that during lens differentiation a number of fibre cell specific membrane proteins change their expression profiles. In this study we have investigated how the profiles of the two most abundant fibre cell membrane proteins AQP0 (formerly known as Major Intrinsic Protein, MIP) and MP20 change as a function of fibre cell differentiation. While AQP0 was always found associated with fibre cell membranes, MP20 was initially found in the cytoplasm of peripheral fibre cells before becoming inserted into the membranes of deeper fibre cells. To determine at what stage in fibre cell differentiation MP20 becomes inserted into the membrane, sections were double-labelled with an antibody against MP20, and propidium iodide, a marker of cell nuclei. This showed that membrane insertion of MP20 occurs in a discrete transition zone that coincided with the degradation of cell nuclei. To test the significance of the membrane insertion of MP20 to overall lens function, whole lenses were incubated for varying times in a solution containing either Texas Red-dextran or Lucifer yellow as markers of extracellular space. Lenses were fixed and then processed for immunocytochemistry. Analysis of these sections showed that both tracer dyes were excluded from the extracellular space in an area that coincided with insertion of MP20 into the plasma membrane. Our results suggest that the insertion of MP20 into fibre cell membranes coincides with the creation of a barrier that restricts the diffusion of molecules into the lens core via the extracellular space.

Keywords: MP20; AQP0; adhesion; membrane junctions; fibre cells; lens

1. Introduction

Cell differentiation involves the acquisition of different cell properties through a series of morphological and molecular changes that culminates in the production of a terminally differentiated cell type (Polakowska and Haake, 1994). In the lens, terminally differentiated fibre cells are derived from equatorial epithelial cells. These cells undergo a process of differentiation in which they dramatically elongate, lose their cellular organelles and nuclei, and express a number of fibre-specific proteins. This differentiation pathway shares elements with apoptosis (Dahm, 1999), yet the fibre cells do not die but are instead internalized by the continual addition of new layers of differentiating fibre cells at the lens periphery. Hence, the lens exhibits a highly ordered spatial gradient of cell age and differentiation, which makes it useful for studying how protein expression patterns change during differentiation.

Indeed several fibre-specific proteins such as the connexins (Cx46 and Cx50) (Lin et al., 1997; Jacobs et al., 2001), the major intrinsic protein (AQP0) (Lo and Kuck, 1990), and cytoskeletal elements (Beebe et al., 2001) have all been shown to undergo specific changes in expression and processing during the course of lens differentiation. This report primarily focuses on the differentiation-dependent changes that occur in the distribution of the second most abundant lens membrane protein, MP20 (Louis et al., 1989). Despite its relative abundance, the function of MP20 in the lens is still not definitively known. MP20 has been implicated as a component of membrane junctions between lens fibre cells (TenBroek et al., 1992; Arneson and Louis, 1998), and more recently it was shown that MP20 acts as a ligand for galectin-3 (Gonen et al., 2001), a known modulator of cell-to-cell adhesion in other tissues (Barondes

* Corresponding author. Dr Paul Donaldson, Department of Physiology, School of Medical Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand.
E-mail address: p.donaldson@auckland.ac.nz (P.J. Donaldson).

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et al., 1994; Kaltner and Steierstrofer, 1998; Perillo et al., 1998). Furthermore, mutations in MP20 severely disrupt the crystalline architecture of lens fibre cells resulting in complete opacification of the lens (Steele et al., 1997). These results are consistent with a role for MP20 in cell–cell adhesion, however, the precise role of MP20 in lens structure, and its impact on lens function has yet to be determined.

Here we report results that support a role for MP20 as an adhesion molecule in the lens. By comparing the relative distributions of MP20 and AQP0 as a function of fibre cell differentiation we have found that MP20, but not AQP0, becomes inserted into the fibre cell membranes at the stage when the cells lose their nuclei. Furthermore, the insertion of MP20 correlates with the formation of a diffusion barrier that restricts the further extracellular movement of molecules into the lens core. These results are consistent with the view that membrane insertion of MP20 may contribute to the interactions between adjacent fibre cells that restrict the movement of molecules into the lens via

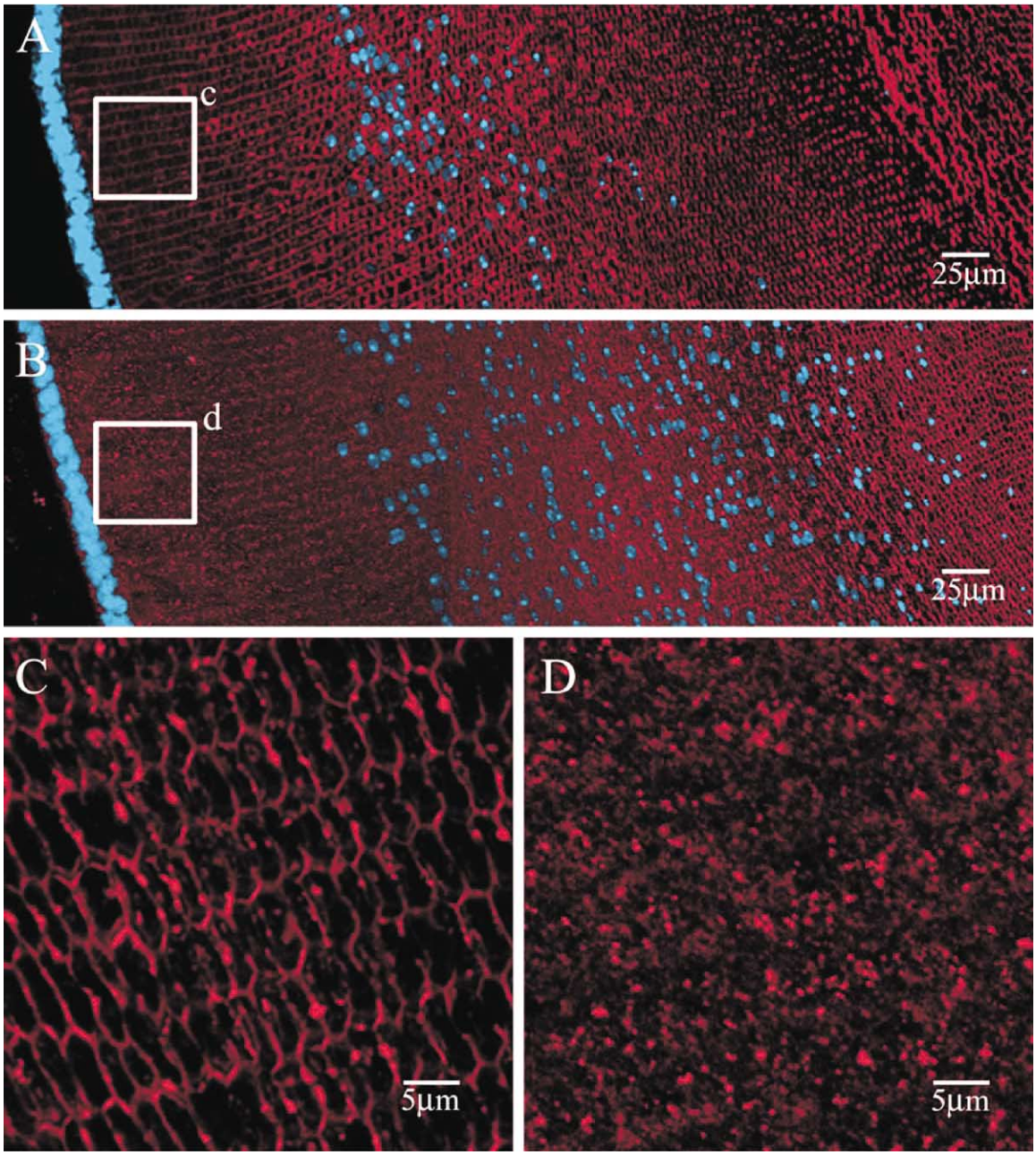

Fig. 1. Comparison of AQP0 and MP20 cellular distribution patterns in the rat lens. Equatorial sections showing an overview of the distribution of the membrane protein (red) AQP0 (A) and MP20 (B) using cell nuclei (blue) as a marker for fibre cell differentiation. (C) Membrane localisation of AQP0 in the peripheral cortex as indicated by box c. (D) Cytoplasmic vesicular localisation of MP20 in the peripheral cortex as indicated by box d.

the extracellular space. The implications of this phenomenon for lens function are discussed.

2. Materials and methods

2.1. Reagents

The carboxyl tail specific MP20 antibody was a gift from Professor Charles Louis, University of Minessota, St Paul (Arneson and Louis, 1998). Anti-AQP0 antibodies were obtained from Alpha Diagnostic International (San Antonio, TX, USA) and have been used previously by others (Shiels and Bassnett, 1996). Phosphate buffered saline was prepared fresh from PBS tablets (Sigma Chemical Company, Australia). Unless otherwise stated all other chemicals were from Sigma.

2.2. Immunocytochemistry

Lenses were extracted from 21-day old weaner rats immediately following death, and fixed with 0·75% paraformaldehyde at room temperature for 24 hr using protocols developed in our laboratory (Jacobs et al., 2003). Alternatively, for dye-uptake studies, freshly extracted lenses were first incubated in culture medium (M199, Sigma) that contained 1·25 mg/ml of either Texas Red-dextran or Lucifer yellow (Molecular Probes, Eugene, OR, USA), for 18 hr, before being fixed as above. From this step onwards all lenses were treated similarly as follows. Lenses were washed 3 times for 10 min in PBS and cryo-protected by incubation for 1 hr in a 10% sucrose solution in PBS, followed by incubation in 20% sucrose solution in PBS for 1 hr. Both incubations were at room temperature. Finally, lenses were incubated overnight at 4°C in a 30% sucrose solution in PBS as a cryoprotectant. For sectioning, whole lenses were mounted in either an equatorial or axial orientation on pre-chilled chucks and encased in Tissue-Tek O.C.T. compound. Lenses were cryosectioned at −18°C on a cryostat (CM3050, Leica, Germany) using disposable blades (S-35; Feather Safety Razor Co., Japan). 10 μm thick equatorial sections, 16 μm thick axial sections, or 40 μm thick equatorial sections from lenses which had been incubated with either Texas Red-dextran or Lucifer yellow, were transferred onto poly-L-lysine coated microscope slides and washed three times in PBS. Sections were incubated in blocking solution (3% bovine serum albumin, 3% fetal calf serum in PBS) for 1 hr at room temperature and washed three times for 5 min in PBS. The tissue was incubated in a solution of rabbit anti-AQP0 (Shiels and Bassnett, 1996) or rabbit anti-MP20 (Arneson and Louis, 1998) at a dilution of 1:100 in blocking solution for 2 hr at room temperature. Slides were washed 3 × 5 min in PBS and incubated for 1·5 hr, in the dark at room temperature, with anti-rabbit immunoglobulins conjugated with Alexa 488, Alexa 568 (Molecular Probes, Eugene, OR, USA) or Indodicarbocyanine (Cy5) (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Cell nuclei were stained with 100 μM propidium iodide for 5 min, at room temperature. 10 μl of anti-fade reagent (Citifluor AF1, Leicester, UK) was added to the slides, and images of each chromophore-staining pattern were recorded digitally using a laser scanning confocal microscope (Leica TCS 4D, Heidelberg, Germany), then pseudo-coloured and combined using Adobe Photoshop software.

2.3. Membrane biochemistry

Sheep lenses were obtained from the local abattoir and stored at −80°C until use. Typically, around 100 lenses were thawed and decapsulated with a sterile surgical blade and dissected into three fractions: outer cortex, inner cortex, and inner core. All three samples were homogenized in 10 mM Tris pH 8, 5 mM EDTA, 5 mM EGTA. Crude membranes were prepared from each of the three fractions by differential centrifugation as previously described (Gonen et al., 2001). Briefly, homogenized tissue was centrifuged in a Sorvall 5C centrifuge and an SS34 rotor at 12 000 rpm for 20 min. The pellet was collected and washed once with 4 M urea, 5 mM Tris pH 9·5, 2 mM EDTA, 2 mM EGTA and once with 20 mM NaOH, and centrifuged using a Sorvall Discovery 100S using an SW65 rotor. Pellets were resuspended into 5 mM Tris pH 8, 2 mM EDTA, 2 mM EGTA at a concentration of 1 mg/ml each as determined by using the BCA protein assay kit according to manufacturer's instructions (Pierce, IL, USA).

Proteins were separated on 17·5% SDS-PAGE gel slabs according to manufacturer's instructions using a Mini-PROTEAN II Electrophoresis Cell (Bio–Rad, Hercules, CA, USA). Proteins were solubilized without boiling in sample buffer containing 15 mM Tris pH 6·8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0·01% bromophenol blue. Silver staining was used to visualize proteins in the gel.

Alternatively, the relative amounts of AQP0 and MP20 in the membrane fractions were estimated by dot-blot analysis using anti-AQP0 (Shiels and Bassnett, 1996) and anti-MP20 (Arneson and Louis, 1998), respectively. Equal aliquots were spotted onto Hybond-C pure nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL, USA) and dried. After washing with MilliQ water, the nitrocellulose membranes were blocked overnight at 4°C in 1% BSA, 2 mM EDTA, 0·1% Tween20 in TBS. MP20 was detected with a 1:1000 dilution of a carboxyl tail specific antibody kindly donated by Professor Charles Louis, University of Minessota, St Paul (Arneson and Louis, 1998). Anti-AQP0 antibodies obtained from ADI (Shiels and Bassnett, 1996) were used at a dilution of 1:1000. Bound antibodies were detected with biotinylated secondary antibodies at 1:1000 and streptavidin-biotin at 1:2000 according to manufacturers instructions (ECL kit, Amersham Life Science).

3. Results

3.1. A comparison of MP20 and AQP0 protein distributions

The distribution of MP20 and AQP0 in the lens was investigated in both rat and sheep lenses. The precise mapping of changes in MP20 distribution as a function of lens differentiation was performed using rat lenses since we have optimized our protocols to correlate membrane protein distribution with tissue morphology in this species (Jacobs et al., 2003). Biochemical experiments were performed with sheep lenses since their larger size facilitated a more accurate dissection into different regions prior to preparation of cell membranes (Kistler and Bullivant, 1987). A comparison of AQP0 and MP20 immunolabelling patterns indicates that although AQP0 and MP20 are both widely expressed in the lens, their spatial distributions are strikingly different (Fig. 1). AQP0 revealed a regular membrane staining pattern (Fig. 1(A)). In contrast, the MP20 signal was characterized by a more random cytoplasmic labelling in peripheral fibre cells, which redistributed to a regular membrane staining with distance into the lens (Fig. 1(B)). Higher magnification images show that in the peripheral cortex, AQP0 is localized clearly to the fibre cell membrane (Fig. 1(C)), while MP20 seems to be associated with vesicles in the cytoplasm of nucleated fibre cells (Fig. 1(D)).

A redistribution of MP20 from cytoplasm to cell membrane was also observed upon analysis of membrane fractions derived from sheep lenses (Fig. 2). While cell membranes prepared from the outer cortex, inner cortex and core of the lens showed minimal changes in AQP0 levels by SDS-PAGE analysis (except for an increase in cleavage of AQP0 to MP24 and MP22 which had been observed before (Bok et al., 1982; Alcala et al., 1987)), the amount of membrane associated MP20 increased towards the lens core by 29% (Fig. 2(A)). Dot blots of membrane aliquots probed with anti-AQP0 and anti-MP20, respectively, also revealed similar levels for the former and a 20% increase for the latter (Fig. 2(B)). The biochemical analysis thus supports a significant redistribution of MP20 into the plasma membrane albeit not to the extent that we would have expected from the immuno-fluorescence images. Upon reflection, however, it seems unlikely that the dissection of outer and inner cortex would have accurately matched the transition zone for MP20, and that plasma membrane fragments could have been totally separated from microsomes – both deficiencies having the effect of reducing the difference that could be measured by SDS-PAGE analysis.

Taken together, both immunolabelling and biochemical results support a redistribution of MP20 from predominantly vesicles in the outer cortex to the plasma membrane in the older fibre cells.

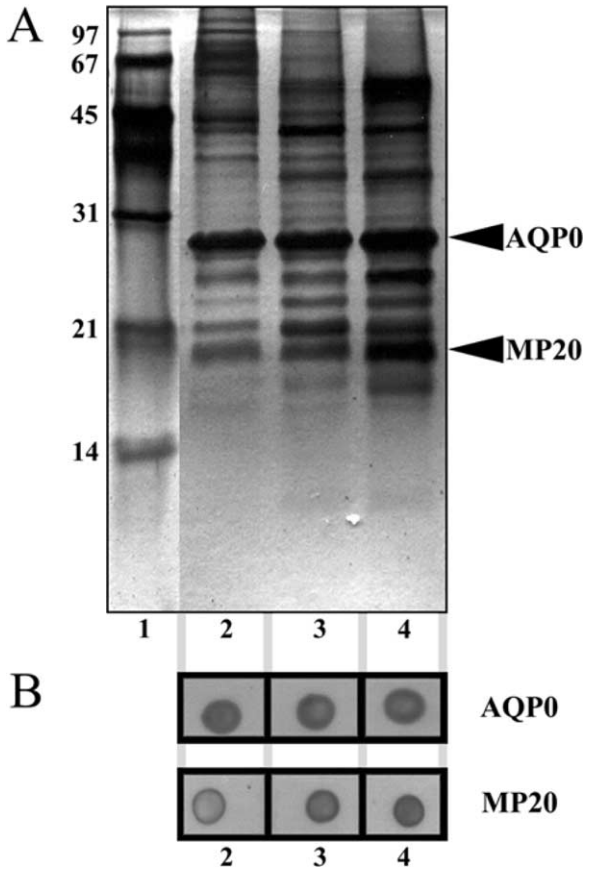

Fig. 2. Comparative presence of AQP0 and MP20 in sheep lens membrane fractions. (A) Silver stained SDS-PAGE gel of urea stripped sheep lens membrane proteins. Lane 1: molecular weight markers; Lane 2: membrane proteins from the outer cortex; Lane 3: membrane proteins from inner cortex; Lane 4: membrane proteins from lens core. The amount of AQP0 is approximately constant in the different fractions, but MP20 increases in membrane fractions prepared from older fibre cells. (B) Dot blots of the same membrane fractions probed with AQP0 and MP20 specific antibodies also reveal a trend of increasing levels of MP20 towards the lens core region.

3.2. Differentiation-dependent insertion of MP20 in fibre cell membranes

In order to more accurately relate this redistribution of MP20 to the membrane to a particular stage of fibre cell differentiation, the immunolabelling pattern of MP20 was examined in lens axial sections in which cell nuclei were also labelled (Fig. 3(A)). Closer inspection shows that MP20 labelling is clearly intracellular in the younger fibre cells of the cortex (Fig. 3(B)), but redistributes to the plasma membranes as the cells mature (Fig. 3(C) and (D)). Furthermore, the redistribution from the cytoplasm to the plasma membrane occurs over only a small number of cell layers (Fig. 3(C)). Interestingly, MP20 seems to be inserted into the plasma membrane at the specific stage of fibre cell maturation that is characterized by the loss of the cell nuclei. This abrupt redistribution of MP20 to the membrane has not been reported before and raises new questions about its function in the lens.

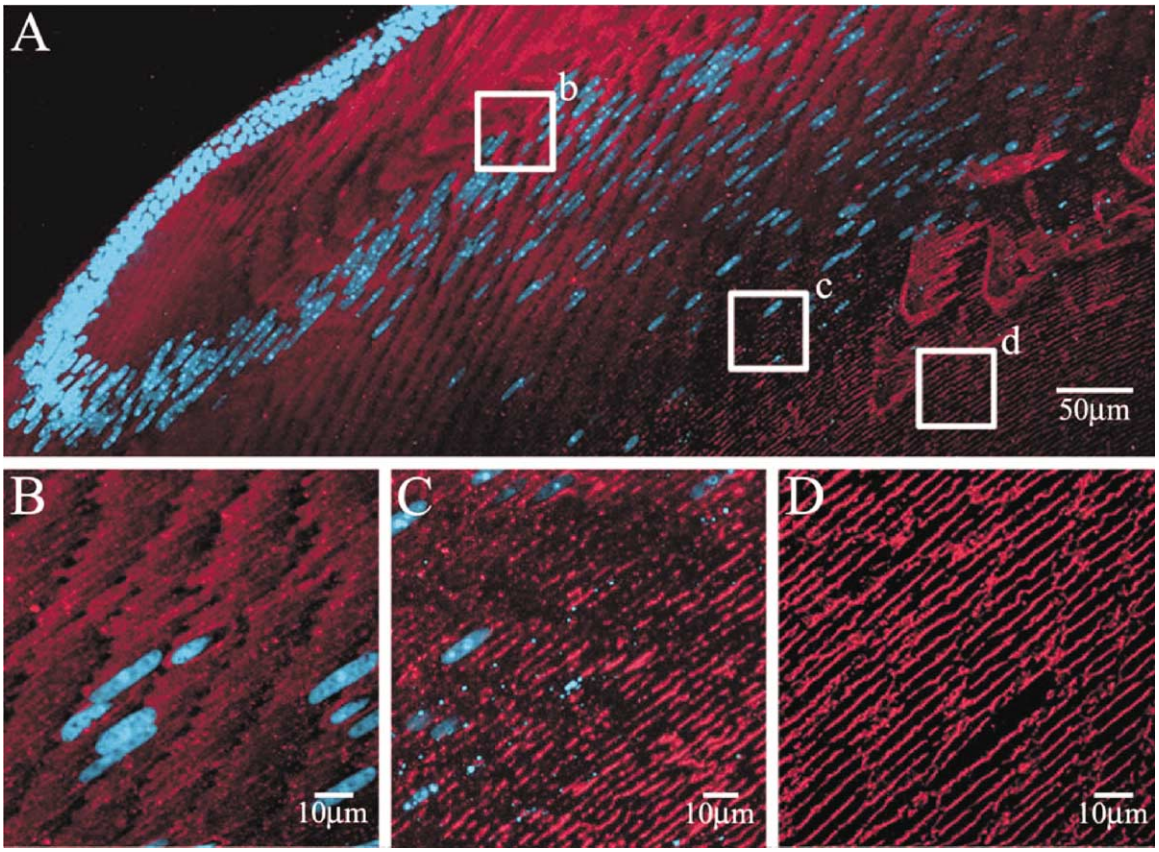

Fig. 3. Differentiation-dependent changes in the cellular distribution of MP20. (A) Axial section through a rat lens showing an overview of the distribution of MP20 (red) and cell nuclei (blue). (B) Random cytoplasmic distribution with bright vesicles of MP20 in area indicated by box b. (C) Redistribution of MP20 from cytoplasm to membranes in area of nuclear degradation as indicated by box c. (D) Regular membrane localisation of MP20 in area indicated by box d.

3.3. Co-location of MP20 insertion and extracellular space restriction

MP20 has previously been implicated as an adhesion protein (TenBroek et al., 1992; Arneson and Louis, 1998; Gonen et al., 2001). If this were correct, the insertion of MP20 into the membrane of mature fibre cells would increase adhesion between the fibre cells, and one would expect that the extracellular space would abruptly become more tortuous, restricting diffusion of molecules deeper in the lens. To test this hypothesis, whole lenses were incubated under organ culture conditions in the presence of two fluorescent extracellular space markers, Texas Red-dextran (MW 10 kDa) and Lucifer yellow (MW 456 Da), for varying times (Fig. 4). Regardless of the incubation period (2–18 hr) Texas Red-dextran diffusion into the lens only occurred over a distance of some 400 μm in from the capsule (Fig. 4(A)–(C)). This consistency in the depth of tracer penetration observed at all three time points indicates that Texas Red-dextran movement via the extracellular space is not diffusion limited, but restricted by a physical barrier. In support of this, the extracellular diffusion of the smaller molecular weight dye Lucifer yellow also became restricted at around the same depth (Fig. 4(D)). This indicates the barrier to extracellular space diffusion has a molecular weight cut-off of at least 456 Da. Subsequent immunolabelling with MP20 antibodies of sections derived from a lens incubated in Texas Red-dextran for 18 hr indicates that the barrier to extracellular diffusion overlaps the zone where MP20 is inserted into the membrane (Fig. 5). This observation suggests the possibility that the insertion of MP20 into the cell membrane contributes directly or indirectly to the formation of an extracellular diffusion barrier.

4. Discussion

In this study we have investigated the relative cellular distributions of the two most abundant membrane proteins in the lens, MP20 and AQP0. While AQP0 is associated with fibre cell membranes throughout the lens, the distribution of MP20 changes as a function of fibre cell differentiation. Localized initially in the cytoplasm of the peripheral fibre cells, MP20 is eventually inserted into the membranes in the deeper, more differentiated fibre cells, a phenomenon that coincides with an abrupt restriction of extracellular space dye diffusion. This implies that

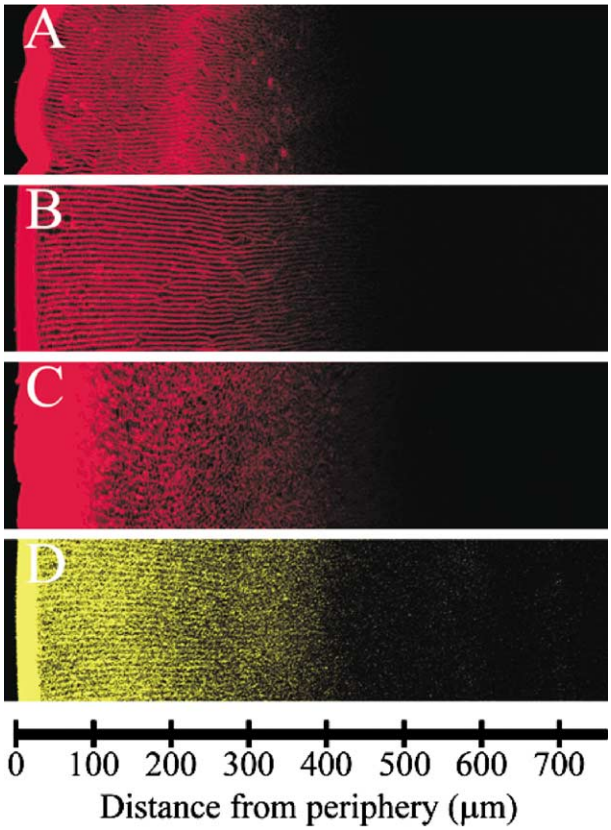

Fig. 4. Extent of extracellular tracer dye diffusion in the cortex region. Rat lenses were organ cultured in medium containing either Texas Red-dextran (MW 10 kDa) or Lucifer yellow (MW 456 Da), and the extent of dye permeation into the lens was visualized in equatorial cryosections. (A)–(C) Extracellular diffusion of Texas Red after 2- (A), 4- (B), and 18-hr (C) incubation periods. (D) Extracellular diffusion of Lucifer yellow after a 4-hr incubation period. Penetration of both dyes at all time points showed a similar drop-off in intensity at around 400 μm, indicating that dye diffusion into the lens via the extracellular space was not limited by diffusion rates but by a physical barrier.

the young, nucleated lens fibre cells manufacture all the MP20 they require while gene expression is possible. They appear to store the majority of MP20 in a pool of cytoplasmic vesicles. Then at a stage in fibre cell differentiation that coincides with the loss of the cell nuclei, MP20 inserts into the plasma membrane.

This observed change in the cellular distribution of MP20 appears to be but one of a number of mechanisms adopted by the lens to compensate for the inability of its older anucleate fibre cells to synthesize new membrane proteins. For example, the fibre cell gap junction protein, Cx50, while always present in the membrane, undergoes a differentiation-dependent cleavage that, like the insertion of MP20, coincides with the loss of cell nuclei (Kistler and Bullivant, 1987; Lin et al., 1997). This cleavage event removes the cytoplasmic tail of Cx50 thereby rendering the gap junctions pH-insensitive. This change is thought to preserve inter-cellular communication between older fibre cells in the acidic lens core (Lin et al., 1998). Another example is the facilitative glucose transporter, GLUT3, which also undergoes a differentiation-dependent insertion into fibre cell membranes from a cytoplasmic pool (Merriman-Smith et al., 2003). This high affinity glucose transporter is thought to mediate the uptake of glucose from the tortuous extracellular space into the fibre cells. However, GLUT3 insertion occurs at an earlier stage of fibre cell differentiation than that observed for MP20, suggesting that signals responsible for the insertion of these two membrane proteins may be different. Taken together, it appears that as fibre cells mature and the ability to synthesize new membrane proteins is lost, the lens uses other mechanisms such as post-translational cleavage or transport of proteins to the cell membrane to meet the physiological challenges associated with being buried deeper in the lens mass.

While the functions of Cx50 and GLUT3 are relatively self-evident, the role of MP20 has remained somewhat uncertain. Previous reports have shown that MP20 localizes to lens junctional domains (TenBroek et al., 1992; Arneson and Louis, 1998) and is a ligand for galectin-3 (Gonen et al., 2001), a known adhesion modulator in other tissues. Furthermore, MP20 appears to be a member of an emerging gene family that contains the epithelial membrane proteins 1–3 (EMP1-3), and the peripheral myelin protein 22 (PMP22) all of which have been functionally associated with cell-to-cell contacts, and cell differentiation and proliferation (Taylor and Suter, 1996). The view that MP20 may contribute to cell adhesion is supported by our present observation that the membrane insertion of MP20 coincides with an abrupt restriction of extracellular space diffusion. A previous report also showed a similar differentiation-dependent exclusion of the tracer procion yellow (MW 697 Da) from the extracellular space (Rae and Stacey, 1979). Our study is the first to show that this coincides with the insertion of MP20 into fibre cell membranes.

What is the significance of the observed changes in the cellular distribution of MP20 and the coincident restriction of extracellular space diffusion to overall lens function? It has been proposed that the avascular lens operates a microcirculation system that delivers nutrients to, and removes waste products from the deeper-lying fibre cells (Mathias et al., 1997; Donaldson et al., 2001). This delivery of nutrients has been proposed to occur via solute drag that accompanies isotonic fluid transport, which is in turn generated by ion movement through the extracellular space. Thus our finding that extracellular diffusion of molecules becomes restricted at the location where MP20 is inserted into the membrane raises questions about the efficiency of this system in the deeper lens. To specifically address this issue, tracer dye diffusion would need to be further investigated using smaller and more functionally relevant molecules such as glucose or amino acids.

Studies conducted on lenses obtained from *To3* mutant mice suggest that the insertion of MP20 into lens fibre cell membranes plays a critical role in lens transparency (Steele

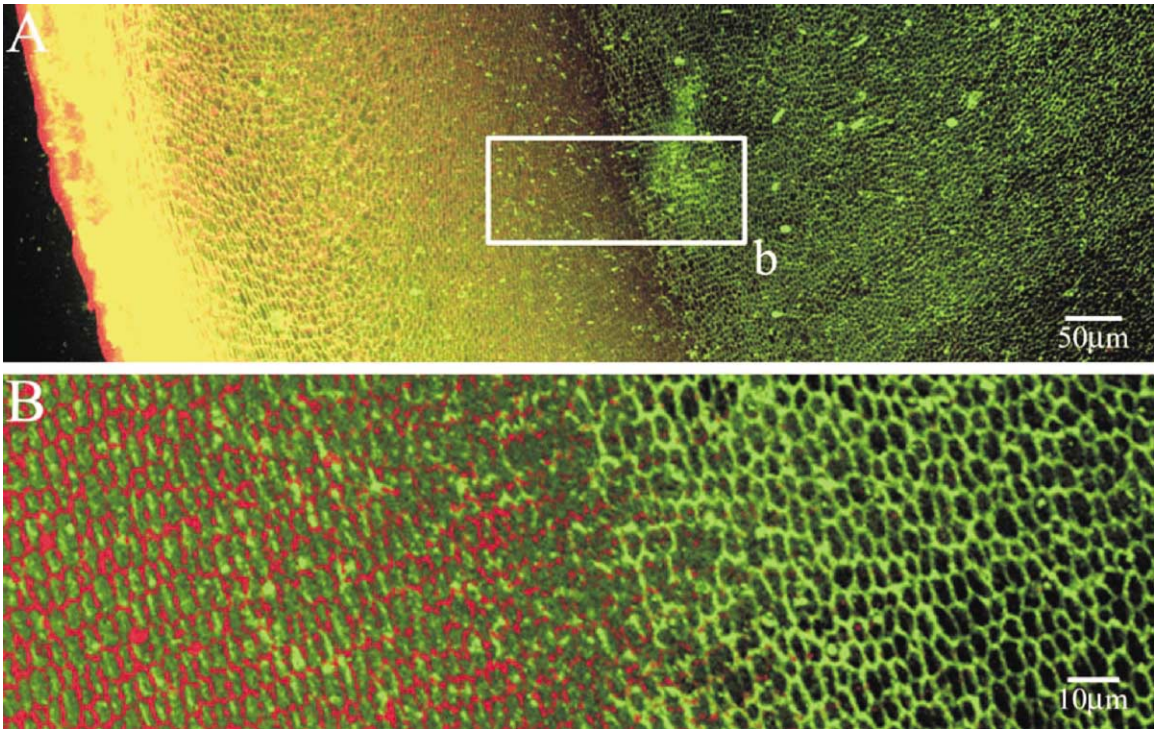

Fig. 5. Spatial co-location of MP20 membrane insertion and dye restriction in rat lenses. (A) Equatorial section showing an overview of the distributions of MP20 (green) and the extracellular marker Texas Red-dextran (red). (B) View of the transition zone from cytoplasmic MP20 to membrane-bound MP20 as indicated by box b in panel A. The Texas Red-dextran signal disappears as MP20 is being inserted into fibre cell membranes.

et al., 1997; Chen et al., 2002). In *To3* mice, a mutation in the MP20 gene results in severe damage to fibre cells in the lens core and nuclear cataract (Steele et al., 1997). In cell lines expressing the *To3* mutation, MP20 fails to insert into the plasma membrane and instead accumulates in the pre-Golgi compartment (Chen et al., 2002). In the normal lens we speculate that in response to the differentiation-related loss of cell nuclei, an as yet undefined signal promotes the insertion of MP20 into the cell membrane. Further experimentation is necessary to discover the nature of this protein trafficking signal and its relevance to lens physiology.

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