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Molecular architecture of the lens fiber cell basal membrane complex

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SUMMARY

Lens fiber cells are transparent, highly elongated, epithelial cells. Because of their unusual length these cells represent a novel model system to investigate aspects of epithelial cell polarity. In this study, we examined the fiber cell basal membrane complex (BMC). The BMC anchors fiber cells to the lens capsule and facilitates their migration across the capsule. Confocal microscopy revealed that bundled actin filaments converge beneath the center of each BMC and insert into the lateral membrane at points enriched in N-cadherin. Two other contractile proteins, caldesmon and myosin, were enriched in the BMC, co-localizing with f-actin bundles. The actin/N-cadherin complex formed a hexagonal lattice, cradling the posterior face of the lens. Removal of the capsule caused the tips of the fiber cells to break off, remaining attached to the stripped capsule. This provided a method for assaying cell adhesion and purifying BMC components. Fiber cell adhesion required Mg^{2+} and/or Ca^{2+} and was disrupted by incubation with β1 integrin antibody. BMC proteins were compared with samples from the neighboring lateral membrane. Although some components were common to both samples, others were unique to the BMC. Furthermore, some lateral membrane proteins, most notably lens major intrinsic protein (MIP), were excluded from the BMC. Western blotting of BMC preparations identified several structural proteins originally found in focal adhesions and two kinases, FAK and MLCK, previously undescribed in the lens. These data suggest that the BMC constitutes a distinct membrane domain in the lens. The structural organization of the BMC suggests a role in shaping the posterior lens face and hence the refractive properties of the eye.

Key words: Lens, Fiber, Actin, Confocal microscopy, Differentiation

INTRODUCTION

Tight junctions at the border of the apical and lateral membranes divide the plasma membranes of epithelial cells into two domains: an apical membrane and a contiguous basolateral membrane. This designation is sufficient to model the transport properties of epithelia but overlooks the fact that some membrane specializations are found exclusively in a third domain, the basal membrane. Specializations of the basal membrane and underlying cytoskeleton are associated with the attachment of epithelial cells to the basal lamina. Although physical barriers (such as tight junctions) do not cordon off the basal membrane domain, this region nevertheless constitutes a structurally and biochemically distinct region. Indeed, it is the basal membrane domain that specifies cell polarity during epithelial development (Wang et al., 1990), and signaling molecules clustered at the basal membrane affect such aspects of cell behavior as proliferation and death (Burridge et al., 1996; Guan 1997).

The interaction of cells with the substratum has been intensively studied in tissue culture. Focal contacts between the ventral surface of cultured cells and the underlying matrix are the paradigm for cell substrate interactions in general. Focal contacts are the points where actin stress fibers terminate. In these regions, clustered integrins bind extracellularly to ligands in the matrix and intracellularly, via linker proteins, to the cytoskeleton. Focal contacts are enriched in signaling molecules that transduce physical and chemical information in the extracellular environment.

Despite their presumed importance, focal contacts are rarely observed in vivo. We have begun to investigate the interaction between lens fiber cells and the lens capsule (the basal lamina of the lens; see Fig. 1) as a novel model for epithelial cell-substrate interactions in vivo. The lens has advantages for such studies. Lens fiber cells are among the longest epithelial cells in the body, with widely separated apical and basal membranes. The lens is a non-innervated, avascular tissue that can be removed from the eye without contamination by other cell types and maintained in organ culture. Furthermore, the transparency of the cells allows microscopical approaches that would not be feasible in other systems.

Despite its relatively small area (<0.1% of the total plasma membrane), the fiber cell basal membrane and associated cytoskeletal elements (here collectively referred to as the basal membrane complex; BMC) are critical for lens function. The BMC anchors the basal membranes of the fibers to the lens capsule. This attachment is strong enough to resist the forces generated during lens accommodation. Fiber cells are a

migratory population of cells. During differentiation, their posterior tips traverse the capsule from the lens equator to the posterior suture (see Fig. 1) often a journey of several millimeters. At the suture, fibers detach from the capsule and dock with cells from the opposite hemisphere of the lens. The BMC ensures proper tracking of the fiber cells across the capsule, timely detachment from the capsule and accurate docking at the suture. Collectively, the BMCs constitute the posterior face of the lens, a critical refractive surface. The radius of curvature of this surface is crucial in determining the focal length of the lens.

We have examined the lens fiber BMC with regard to its role in cell migration, adhesion and potential role in image formation. Our data indicate that this small patch of basal membrane is a dynamic structure that is remodeled as fiber tips traverse the lens capsule. We have identified membrane protein components of the BMC and some of the associated cytoskeletal and regulatory elements. The structural organization of this domain suggests a role in shaping the posterior lens face and hence the refractive properties of the eye. Because the fiber cell basal membrane domain is easily visualized and isolated, it may represent a useful experimental system for more generalized studies of cell/cell and cell/substrate interactions.

MATERIALS AND METHODS

Animals

White Leghorn chicken eggs were obtained from Truslow Farms (Chestertown, MD) and incubated at 38°C. Lenses were removed on embryonic day 10-18 (E10-E18).

Lens slices

Lenses were fixed in 4% paraformaldehyde/PBS for 1 hour and cut into 200 μm slices as described (Bassnett and Mataic, 1997).

Fiber cell migration rate

The rate at which fiber cells migrated across the capsule was estimated by calculating the daily rate of increase in length of the posterior suture during embryonic development (see Fig. 1). Suture length was determined from images of lens slices stained with the lipophilic fluorescent probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) as described (Bassnett, 1995). The rate of increase in suture length was used to calculate the number of cells entering the suture each day. Measuring the size of fiber cell footprints on the posterior capsule allowed an estimate of the speed at which the fibers were migrating across the capsule.

Lens capsule permeability

The permeability of the lens capsule was examined by monitoring the diffusion of a fluorescein-conjugated rabbit IgG (≈160 kDa: Jackson Immunolabs) into the lens. Lyophilized antibody was reconstituted in PBS at 1 mg/ml and dialyzed overnight against PBS to remove preservatives and unconjugated fluorochrome. E18 lenses were incubated in 100 μl of fluorescent antibody and examined by confocal microscopy to determine whether the fluorescent antibody had penetrated the capsule.

Immunofluorescence

Antibodies were applied to fixed, permeabilized lens slices as described (Bassnett and Mataic, 1997). Primary and secondary antibodies were applied at 1:500 dilution. Texas Red-phalloidin (TR-phalloidin; Molecular Probes) was used to visualize the distribution of f-actin. Tissue was incubated for 30 minutes in a 1:100 dilution (in PBS) of a methanolic stock solution (200 U/ml) of TR-phalloidin and washed for 3× 10 minutes in PBS.

Antibodies

Antibodies against the following proteins were used: chicken major intrinsic protein (MIP; from Dr Sue Menko), β1 integrin (JG22; Developmental Studies Hybridoma Bank), caldesmon (Sigma; clone CALD-5), zyxin (Transduction Laboratories; clone 21), vinculin (Sigma; clone VIN-11-5), myosin light chain kinase (Sigma; clone K36), myosin (Sigma; clone MY-21), focal adhesion kinase (Transduction Laboratories; clone 77), paxillin (Transduction Laboratories; clone 349), actin (Sigma; clone AC-40), α-actinin (Sigma, rabbit antiserum), N-cadherin (Sigma; clone GF4), talin (Sigma; clone 84d).

Adhesion assay

Posterior capsules were carefully stripped from the lens. Dissection was performed in modified Ringer's solution of the following composition (mM): NaCl, 113; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$, 1.5; glucose, 6; Hepes, 10; $NaHCO_3$, 20, pH 7.3. Examination of stripped capsules revealed the presence of sheared-off posterior fiber tips on the inner surface of the capsule. An f-actin-specific fluorescent probe, TR-phalloidin, was used to visualize the adherent fiber tips. The dissected capsule was fixed for 30 minutes in 4% paraformaldehyde, washed in PBS and stained for 30 minutes with TR-phalloidin (as described above). In some experiments, lenses were incubated in divalent cation-free solution for 10 minutes before dissecting the capsule. This solution had the following composition (mM): NaCl, 113; KCl, 4.5; EDTA, 2.5; glucose, 6; Hepes, 10; $NaHCO_3$, 20, pH 7.3.

To examine the effect on fiber cell adhesion of JG22, a function blocking chicken β1 integrin monoclonal antibody (Greve and Gottlieb, 1982), lenses were cultured overnight in Eagle's minimum essential medium (supplemented with 5% fetal calf serum, 5% tryptose phosphate broth and gentamycin) containing JG22 (1:100 dilution of ascites fluid). Contralateral lenses were incubated in medium lacking the antibody. Following incubation, posterior capsules were stripped from the lens, stained with TR-phalloidin and viewed by confocal microscopy. Eight images were collected randomly from each capsule preparation. The images were analyzed using Metamorph image analysis software (Universal Imaging Corporation, West Chester, PA) to determine the proportion of capsule area occupied by the severed fiber tips. Data from treated and control contralateral lenses were compared using a paired Student's *t*-test.

Biotinylation

To visualize the protein in the sheared-off fiber tips by SDS-PAGE, a biotinylation method was used. Posterior capsules were dissected from four E15 lenses. A crude, PBS-insoluble, lateral membrane preparation was obtained from the fiber cells remaining after capsule stripping. Some lenses were pre-incubated in Ca^{2+}/Mg^{2+}-free Ringer's solution for 45 minutes before stripping; resulting in detachment of the fiber tips and providing a cell-free capsule for comparison with other biotinylated samples. Samples were biotinylated using a commercial kit (Amersham) according to the manufacturer's instructions. Biotinylated proteins were separated on 7.5% acrylamide gels, transferred to nitrocellulose and probed with horseradish peroxidase (HRP)-conjugated streptavidin. Bound streptavidin-HRP was visualized by enhanced chemiluminescence (ECL; Amersham).

Western blot

Posterior capsules were dissected from 15-20 E15 lenses and solubilized in sample buffer. Samples were separated on 12% acrylamide gels, transferred to nitrocellulose and probed with antibodies against adhesion proteins using ECL (Amersham) to detect bound antibody.

RESULTS

Migration

The cellular organization of a mid-sagittal lens section is shown in Fig. 1A. At the equator, epithelial cells begin to differentiate into lens fiber cells. As the fiber cells elongate, their posterior tips migrate beneath the lens capsule (in the direction indicated by the arrow in Fig. 1A) from the equator (position a in Fig. 1A) to the posterior suture (position d). At the suture, fibers detach from the capsule and interlock with cells extending from the opposite hemisphere of the lens. Differentiation and migration continue throughout life, resulting in an increase in suture length and a commensurate increase in lens volume.

To calculate the migration rate of fiber cells across the capsule, we measured posterior suture length (SL in Fig. 1B) on successive days of development from E10-E17. These data are shown in Fig. 2. The rate of increase in length of the posterior suture was linear at approximately 45 μm/day. Viewed in the mid-sagittal plane, the average thickness of fiber cells (T in Fig. 1B) was 3 μm. The length of the fiber cell footprint on the capsule (FL in Fig. 1B) was 8 μm. The average fiber cell migration rate was thus (ΔSL/T) × FL or (45/3) × 8 = 120 μm/day.

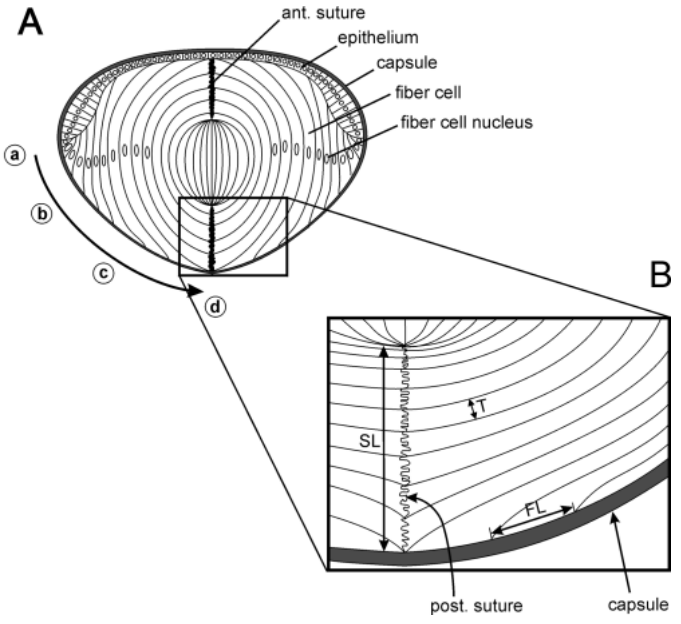

Fig. 1. Cellular organization of a mid-sagittal slice of an embryonic chicken lens. (A) The lens is enveloped by a thick basal lamina, the lens capsule. At the anterior, a monolayer of epithelial cells is located beneath the capsule. At the equator of the lens, epithelial cells differentiate into highly elongated fiber cells. The posterior tips of the fiber cells migrate beneath the posterior capsule (in the direction indicated by the arrow), finally converging on the lens suture at the posterior pole. At the suture, fibers detach from the capsule and interlock with cells from the opposite hemisphere of the lens. As fiber cells traverse the posterior capsule, the organization of the basal membrane complex (BMC) varies. For orientation purposes, we define four arbitrary regions of the posterior capsule (a-d in A). In subsequent figures, these regions are referred to when discussing the properties and organization of the differentiating fiber cells. (B) A higher magnification view of the boxed area from A. In this study, the posterior suture length (SL) was measured at consecutive days of development. In conjunction with measurements of fiber cell thickness (T) and the length of the fiber cell footprint on the capsule (FL) this allowed the migration rate of fiber cells across the capsule to be calculated.

Adhesion

The strength of the adhesive interaction between fiber cells and the capsule was demonstrated by mechanically stripping the capsule from the lens. Examination of the stripped capsule under Nomarski optics revealed that the inner surface was decorated with the sheared-off tips of fiber cells (Fig. 3A). This thin, transparent layer of material consisted of the basal membranes of the fiber cells and underlying cytoskeletal elements (collectively termed the basal membrane complex; BMC). The packing organization of the fiber cell tips on the posterior capsule was visualized by staining isolated capsules with TR-phalloidin to label f-actin. Phalloidin staining revealed a cobblestone pattern of f-actin-rich plaques, each bordered by a narrow actin-free region (Fig. 3B). The plaques were arranged in a hexagonal pattern across most of the posterior capsule. However, near the posterior suture, the packing was less well ordered (data not shown). In the equatorial region, the severed fiber tips were arranged in radially oriented columns (Fig. 3C). Individual fiber cell BMCs in this region were dendritic in appearance, with thin phalloidin-stained processes extending from an actin-rich, central plaque. Relatively large areas of unstained capsule separated the columns of fiber tips from each other. Within a column, gaps in the phalloidin staining presumably marked sites from which fiber cells had been dislodged during dissection.

We tested whether the adhesion of the BMC to the lens capsule was dependent on the presence of Ca^{2+} and Mg^{2+} by pre-incubating lenses in EDTA/Ringer's solution before stripping the capsule from the lens. In control Ringer's solution (containing divalent cations), the stripped capsule was heavily decorated with the densely packed BMCs of the fiber cells (Fig. 4A). However, a 10 minute pretreatment with EDTA/Ringer's solution caused the detachment of the fiber cell BMCs from the capsule. Capsules stripped subsequently from these lenses were completely free of adherent BMCs (Fig. 4B). Treatment with EDTA/Ringer's caused detachment of fiber cells across the entire posterior capsule (from position a-d in Fig. 1A). If

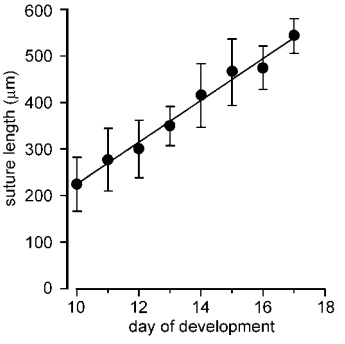

Fig. 2. Change in posterior suture length during embryonic development. Measurements of suture length (SL in Fig. 1B) were made from images of $DiOC_6$-stained lens slices (see text for details). Data represent the mean ± s.d. of measurements from at least six lenses at each time point.

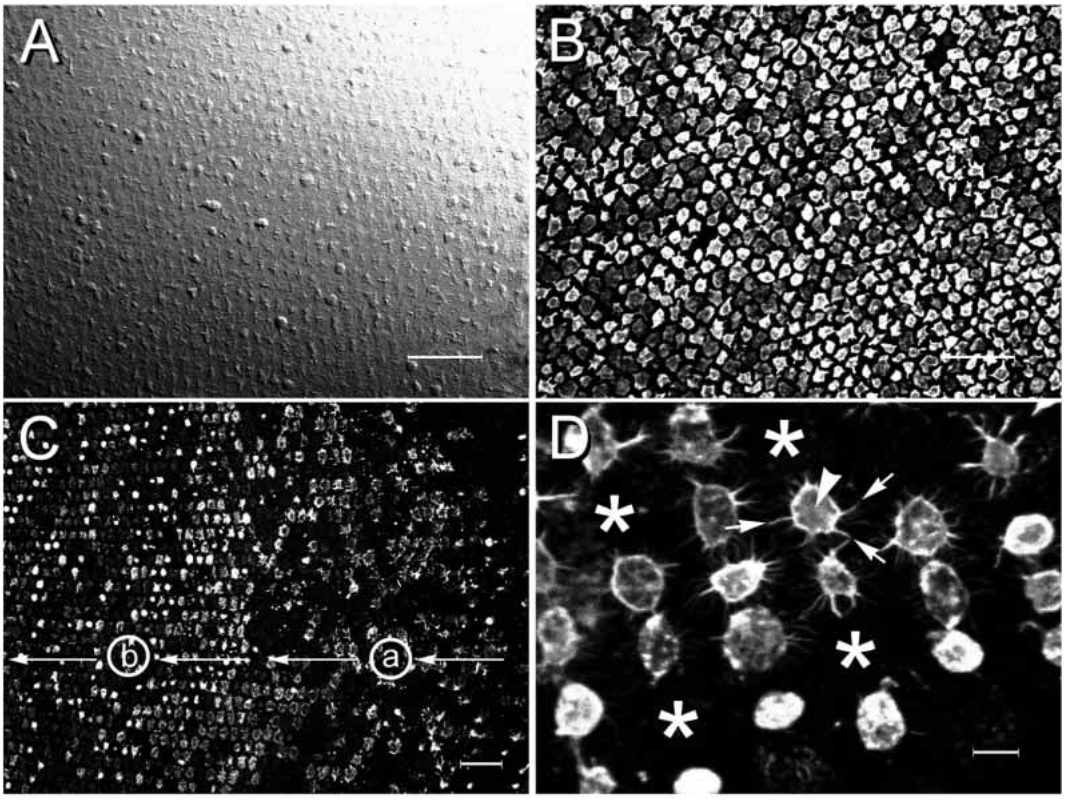

Fig. 3. Isolation of the basal membrane complex (BMC). The fiber cell BMC adheres so strongly to the lens capsule that stripping the lens capsule results in the shearing off of the extreme posterior tips of the fibers. (A) The fiber cell BMCs remain attached to the inner surface of the isolated capsule and can be visualized using Nomarski optics. (B) The BMC contains abundant f-actin that is visualized by TR-phalloidin staining. Phalloidin staining reveals that the BMCs are arranged in a regular hexagonal pattern on the capsule. Each BMC consists of a central f-actin-rich plaque bordered by a narrow unstained region. (C) The packing arrangement of the BMCs varies across the capsule. Near the equator (a; see also Fig. 1A) only 50% of the fiber BMCs remain attached to the isolated capsule. As the fiber cells migrate posteriorly (position b; see Fig. 1A), the BMCs become organized in columns oriented towards the posterior suture (arrows). In this region the BMCs are tightly adherent to the capsule. (D) The arrangement of f-actin in equatorial BMCs (corresponding to position a in Fig. 3C) is unusual. The confocal images reveal a dendritic organization where tendrils of actin (arrows) extend from a central plaque (arrowhead). Note the black borders around each of the brightly stained patches of actin. Gaps in the columnar staining pattern (indicated by *) represent regions from which the fibers are presumed to have detached. Bars: (A, B and C) 50 μm; (D) 10 μm.

lenses were returned to control Ringer's solution for 1 hour following the 10 minute incubation in EDTA/Ringer's solution, the fiber cells reattached to the capsule. Capsules stripped from these lenses were once again heavily decorated with adherent BMCs (Fig. 4C). These experiments demonstrated that attachment of the BMC to the posterior lens capsule was reversibly dependent on the presence of divalent cations. Interestingly, a 10 minute treatment with EGTA/Ringer's solution did not cause BMC detachment, suggesting that removal of Ca^{2+} alone was not sufficient to detach the fibers (data not shown).

Divalent cation-dependent cell/substrate adhesion is often mediated by integrins, and the avian lens expresses several integrin subunits (Menko and Philip, 1995). We visualized the distribution of β1 integrin at the posterior surface of the lens using JG22 (Greve and Gottlieb, 1982), a monoclonal antibody against chicken β1 integrin (Fig. 5). The antibody strongly labeled the fiber cell basal membranes and the adjacent lateral membrane. Although not evident in Fig. 5, faint immunofluorescence was also observed in more distal regions of the lateral membranes, hundreds of micrometers from the capsule. To test directly whether β1 integrin played a central role in fiber cell adhesion to the capsule, we attempted to block adhesion by incubating lenses overnight in JG22. Previous studies demonstrated that JG22 is a function-blocking antibody that interferes with β1 integrin-mediated attachment of myogenic cells (Greve and Gottleib, 1982; Trudel and Holland, 1989), retinal pigment epithelia (Rizzolo and Zhi-Qiang, 1993), and cultured neurons (Halfter and Vonboxberg, 1992). However, in order to block the adhesive interaction between integrins in the BMC and matrix molecules in the capsule, antibodies must first pass through the lens capsule. The capsule is the thickest basal lamina in the body and envelops the lens completely (see Fig. 1). Studies in other species have indicated that the capsule may be impermeable to proteins as large as immunoglobulins (Francois and Rabaey, 1958; Hockwin et al., 1973). In preliminary experiments, therefore, we examined the permeability properties of the embryonic chicken lens capsule by incubating the intact lens in Ringer's solution containing fluorescein-conjugated IgG. We observed that fluorescent IgG molecules readily permeated the capsule and could be detected in the extracellular spaces between fiber cells within 5 minutes (data not shown). Having verified that the capsule was permeable to IgGs, we proceeded to incubate lenses overnight in medium containing anti-β1 integrin (clone JG22). The following day, capsules were stripped from test and control lenses, stained with TR-phalloidin and examined by confocal microscopy (Fig. 6). In control lenses (Fig. 6A), fiber cell adhesion was maintained following overnight incubation. However, in lenses incubated in media containing JG22, there was a striking reduction in the number of fiber tips attached to the stripped capsule. This was most evident over the central regions of the capsule (corresponding to position b-d in Fig. 1A). Near the equator (position a in Fig. 6B and Fig. 1A), the

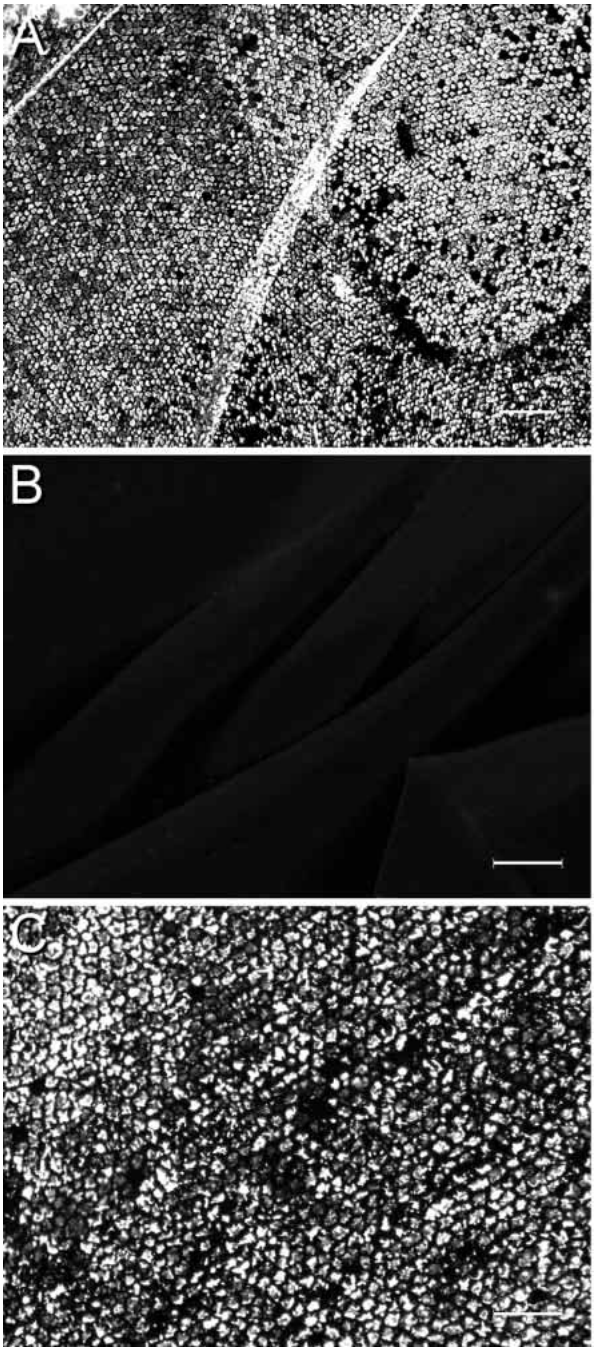

Fig. 4. Effect of divalent cations on adhesion of fiber cells to the posterior capsule. E15 lenses were incubated in control Ringer's solution (A), or divalent cation-free solution (B) for 10 minutes. Some lenses were returned to control Ringer's for 1 hour following divalent cation-free treatment (C). Posterior capsules were removed from the lenses, stained with TR-phalloidin and visualized by confocal microscopy. (A) In control Ringer's, the BMCs of the lens fibers remain attached to the capsule after dissection. (B) Treatment with divalent cation-free solution leads to detachment of the fiber cells. Capsules stripped from these lenses are not decorated with actin-rich plaques. (C) Reintroduction of divalent cations results in reattachment of the fibers. Capsules stripped from these lenses are decorated with actin-rich plaques. Bars: (A and B) 100 μm; (C) 50 μm.

number of attached fiber cells was similar in treated and untreated lenses. The proportion of the isolated capsule covered by BMCs was quantified by analysis of confocal images. In four pairs of lenses (where the contralateral eye served as control), incubation with JG22 caused a significant reduction to 37±30% (mean ± s.d.) of control values (P=0.027). These data support the hypothesis that β1 integrin plays a direct role in attachment of the BMC to the lens capsule.

Molecular composition of the BMC

The lens capsule dissection technique enabled BMC proteins to be purified in a single step, allowing a comparison between BMC components and those of the adjacent lateral membrane. The biotinylated protein profiles of the BMC, the EDTA-treated capsule, and the lateral membrane, are shown in Fig. 7. We previously demonstrated that a brief treatment with EDTA caused fiber cell detachment (see Fig. 4B). Capsule samples collected from EDTA-treated lenses consisted entirely of matrix components. The sparsity of bands in the EDTA-treated capsule preparation (lane C in Fig. 7) indicated that most capsule components were insoluble in the sample buffer. By implication, the bands observed in the BMC sample were derived from the BMC itself rather than the capsule with which it was co-isolated. A comparison of the BMC samples with that of the lateral membrane revealed that although some proteins were found in similar abundance in both preparations, others were enriched in or unique to the BMC. Similarly, some proteins that were abundant in the lateral membrane were excluded from the BMC. This is exemplified by the membrane distribution of lens fiber major intrinsic protein (MIP). MIP is an integral membrane protein and a member of the aquaporin family. It is the most abundant membrane protein in the lens, comprising >50% of the plasma membrane protein (Broekhuyse et al., 1976). We used confocal immunofluorescence microscopy to determine whether this otherwise ubiquitous membrane protein was present in the BMC (Fig. 8). Although readily detected in the neighboring lateral membrane, MIP immunofluorescence was not detected in the basal membrane. Based on the biotinylated protein profiles and the differential distribution of MIP, we conclude that the basal and lateral membranes differ significantly in composition and can be considered distinct domains of the fiber cell plasma membrane.

We used the capsule stripping technique to identify components of the BMC by western blot. A useful paradigm for these studies is the focal adhesion complex which, in cultured cells, forms at sites of contact between cells and substrate. Focal contacts are sites where actin stress fibers terminate and other structural components (e.g. α-actinin, vinculin, talin, tensin, and paxillin) aggregate. A variety of signaling molecules, such as focal adhesion kinase (FAK), p130Cas, and members of the Src family, also localize to focal contacts. We used antibodies against proteins enriched in focal adhesions to screen western blots of lens BMC preparations (Fig. 9). Surprisingly, several characteristic components of focal adhesions were not detected, including α-actinin, zyxin, vinculin, and talin, suggesting that either these proteins were not components of the BMC or that they were lost during the stripping procedure. Actin was detected by western blot,

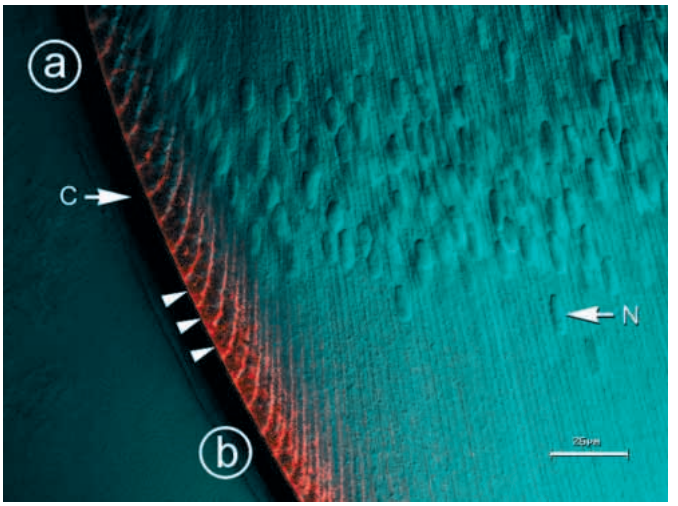

Fig. 5. Distribution of β1 integrin at the posterior surface of the lens. β1 integrin was localized by immunofluorescence confocal microscopy in midsaggital lens slices. This micrograph is from the region immediately below the lens equator (for location of a and b see Fig. 1A). The β1 integrin (red) is present in the BMCs (arrowheads) and adjacent lateral membranes of the fiber cells. The lens capsule (C) and fiber cell nuclei (N) are also visible in the merged Nomarski image (blue). Bar, 25 μm.

consistent with the phalloidin staining noted earlier (Fig. 3). Caldesmon, a contractile protein, was a prominent BMC component, as was paxillin. We also detected two kinases, myosin light chain kinase (MLCK) and FAK. Finally, phosphotyrosine-containing proteins are often concentrated at focal contacts (Schlaepfer and Hunter, 1998). We detected multiple phosphotyrosine-containing species in the BMC, two of which may correspond to MLCK and FAK, which are both phosphorylated on tyrosine residues.

Structural organization of the BMC

The capsule stripping technique is an invasive procedure likely to disturb the organization of actin filaments or other structural elements in the BMC. For morphological analyses, therefore, f-actin was visualized in fixed, intact lenses stained with TR-phalloidin. We observed that some fiber cells were permeable to TR-phalloidin in the absence of detergent treatment. These phalloidin-stained cells were isolated optically from the surrounding, unstained, transparent tissue. Extended focus confocal microscopy was used to visualize the organization of f-actin in the BMC and lateral membranes of these cells (Fig. 10). The f-actin was distributed immediately beneath the lateral membranes of the fiber cells. In the BMC, f-actin was particularly abundant, forming complex, rosette-like structures beneath the basal membrane, at the point of contact with the capsule.

It was apparent from the extended focus images that f-actin was organized differently beneath the basal and lateral membranes. The differential organization of f-actin was studied further in fully permeabilized lenses or slice preparations (Fig. 11). We also examined the distribution of N-cadherin, a cell adhesion protein, because the organization of this molecule beneath the lateral and basal membranes appeared to parallel that of f-actin. The organization of f-actin and N-cadherin in the BMC was visualized en face in

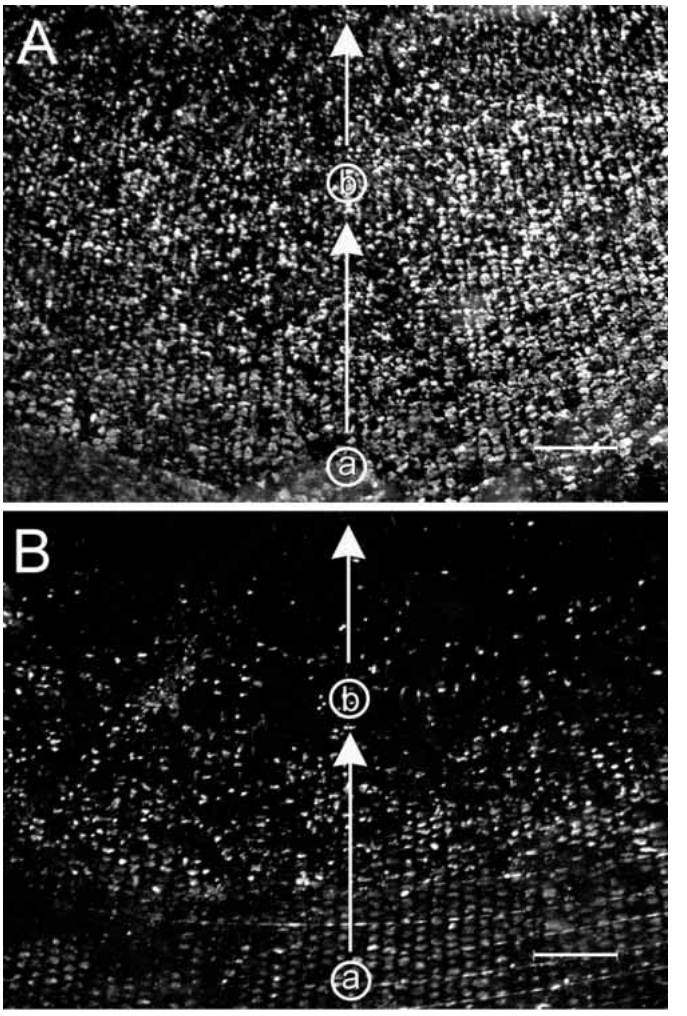

Fig. 6. β1 integrin antibody blocks fiber cell adhesion to the posterior lens capsule. Lenses were incubated overnight in the absence (A) or presence (B) of JG22, a monoclonal antibody against β1 integrin. In the presence of the antibody, there was a significant decrease in the number of BMC's remaining attached to the capsule after dissection. Fiber cell detachment was most marked in the central region of the capsule (b-d in Fig. 1A). In the equatorial region (a) the number of attached BMCs was similar in treated and untreated lenses. Bars: (A and B) 50 μm.

permeabilized intact lenses. Viewed from this aspect, the fiber cell basal membranes appeared as regular hexagons. N-cadherin immunofluorescence was strongest in regions were the faces of two adjacent hexagons made contact (arrowed in Fig. 11A). In contrast, there was relatively little N-cadherin immunofluorescence at the cell vertices, the point at which three adjacent hexagons made contact. Similarly, f-actin was concentrated in two or three discrete foci midway along each of the six lateral faces (arrowed in Fig. 11B) and relatively sparse at the vertices. Bundled actin filaments projected from the f-actin/N-cadherin rich regions on the lateral faces and converged beneath the center of each basal membrane. The organization of f-actin and N-cadherin elsewhere along the length of the fiber cell was strikingly different from that in the BMC. Cross-sectioned at their midpoint, fiber cells had a flattened hexagonal profile with two broad sides (large arrows

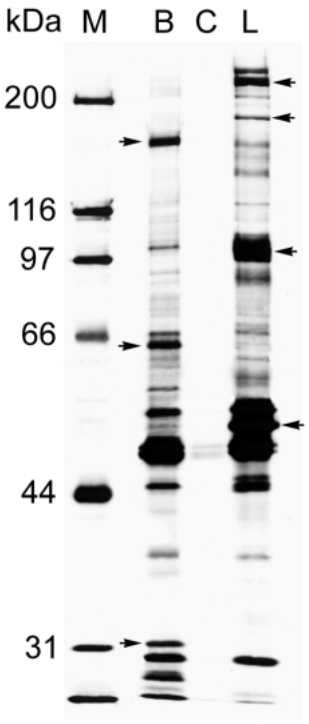

Fig. 7. The basal membrane complex represents a distinct membrane domain. Biotinylated BMC proteins from the isolated posterior capsule (B), EDTA-treated capsule (C), or a lateral membrane preparation (L) were separated by SDS-PAGE and visualized using streptavidin-HRP and ECL. Although the basal and lateral membranes share some components, many proteins are unique to one domain or the other (arrowed). The EDTA-treated capsule (C) contains very few soluble components, demonstrating that the proteins in lane B originate from the fiber cell BMC rather than the extracellular matrix. M, molecular mass marker.

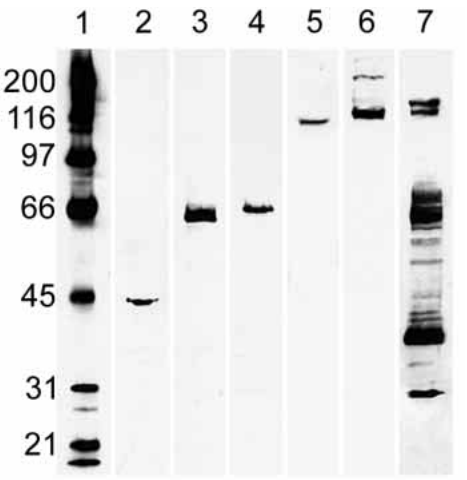

Fig. 9. Western blot analysis of BMC components. 15-20 posterior capsules from E17 chicken lenses were used for each lane. Lanes: 1, biotinylated marker; 2, actin; 3, caldesmon; 4, paxillin; 5, focal adhesion kinase; 6, myosin light chain kinase; 7, phosphotyrosine.

in Fig. 11C,D) and four short sides (small arrows in Fig. 11C,D). The cells were stacked upon each other in radial columns. The organization of f-actin and N-cadherin beneath the lateral membranes in this region of the cell was the reciprocal of that observed in the BMC. N-cadherin was distributed throughout the lateral membranes of the fiber cells but was particularly enriched at the cell vertices (Fig. 11C). Similarly, in cross-sectioned fibers, f-actin was concentrated beneath the vertices of the lateral membranes (Fig. 11D).

We examined the distribution of a second contractile protein, caldesmon, which was initially identified in stripped capsules by western blot (Fig. 9). Caldesmon immunofluorescence was particularly strong in the BMC (Fig. 12A). At higher

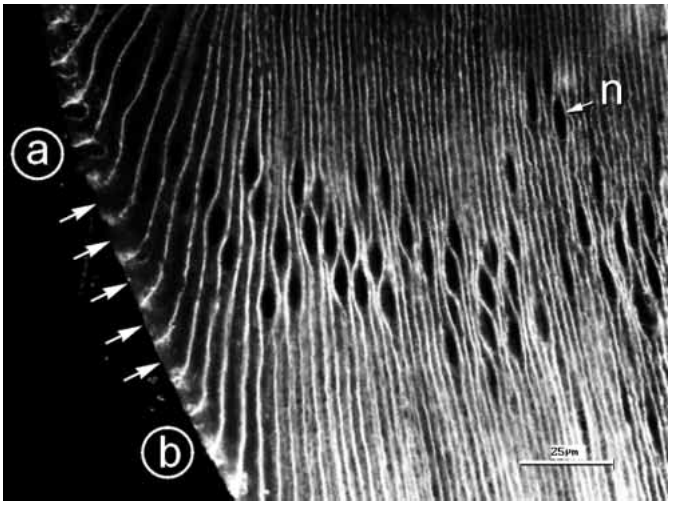

Fig. 8. Localization of the lens major intrinsic protein (MIP) in E15 lens slices. Lens slices were incubated with an antibody against chicken MIP and visualized by confocal microscopy. MIP is an abundant integral membrane protein in the lens and is readily detected in the lateral membranes of the fiber cells. MIP immunofluorescence is not detected in the fiber cell BMC (arrows) suggesting that components of the lateral and basal membrane domains are not free to mix. *n* = fiber cell nucleus. Bar, 25 μm.

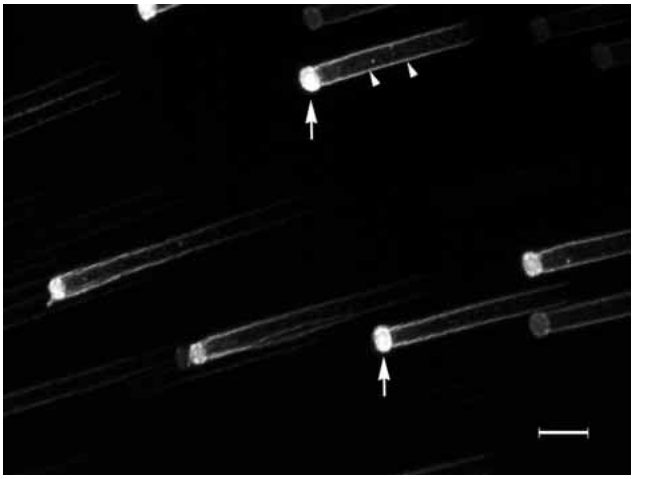

Fig. 10. Distribution of actin beneath the lateral and basal membranes of individual lens fibers. Intact lenses were fixed and stained with TR-phalloidin without detergent permeabilization (see text for details). Occasional lens fiber cells in the intact tissue are stained by the TR-phalloidin. The f-actin is concentrated beneath the lateral membrane of the ribbon-like fiber cells (arrowheads) and is especially enriched in the BMC, where it is organized into complex, rosette-like structures (arrows). Bar, 25 μm.

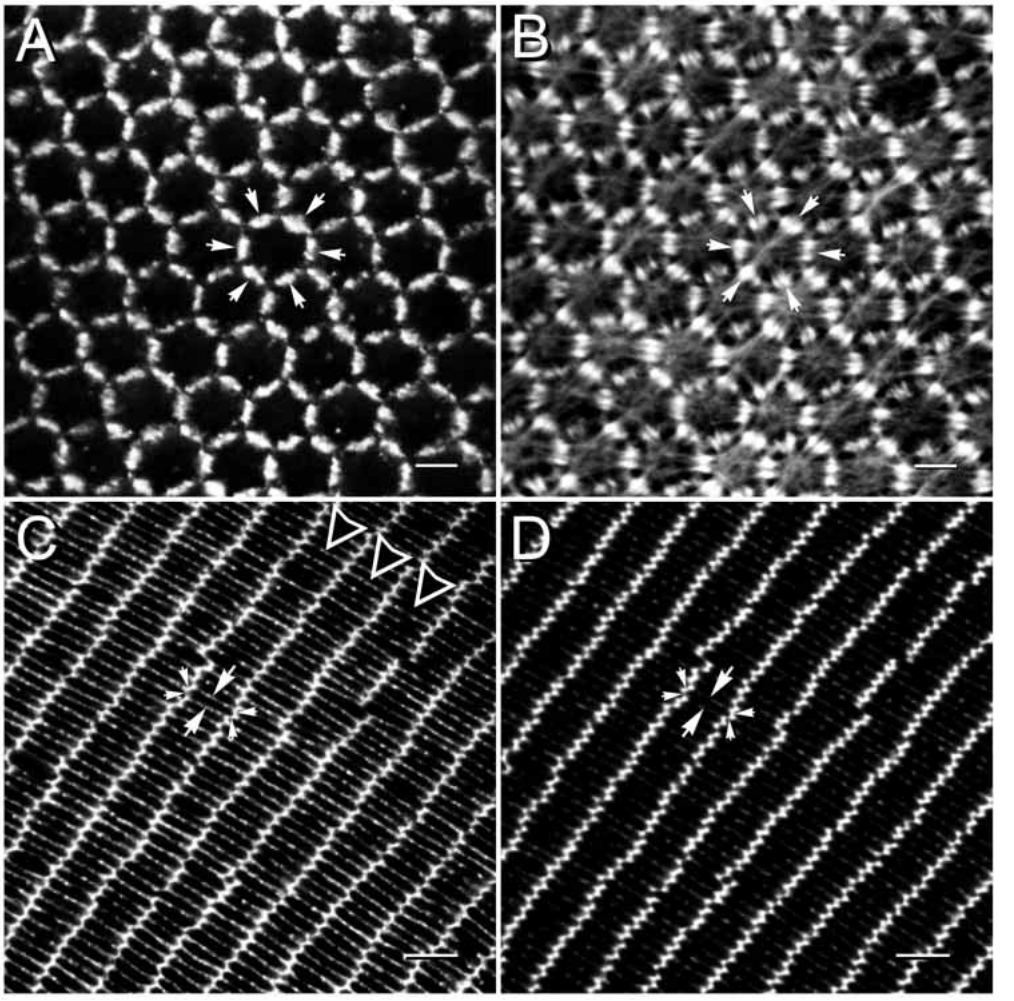

Fig. 11. Arrangement of N-cadherin and f-actin in the BMC and beneath the fiber cell lateral membrane in double stained lens preparations. (A) En face view of N-cadherin immunofluorescence in the BMC imaged through the overlying capsule. The footprint of the fiber cells on the capsule is a regular hexagon. The baso-lateral margin of an individual fiber cell is indicated by arrows. N-cadherin immunofluorescence is strongest at the midpoint of each hexagonal face. (B) Organization of f-actin in the same region as shown in A. Bundles of actin filaments originate from two or three strongly stained foci midway along each face of the hexagonal basal membrane. These regions were also stained by the N-cadherin antibody (compare A and B). Actin filaments originating from the lateral membrane converge beneath the center of the basal membrane. (C) Cross-sectioned at their midpoint lens fiber cells have flattened hexagonal profiles with two broadsides (large arrows), oriented parallel to the lens surface, and four short sides (small arrows). The ribbon-like cells form radial cell columns (arrowheads) oriented perpendicular to the lens surface. N-cadherin immunofluorescence is distributed throughout the lateral membrane but is strongest at the cell vertices. D. Filamentous actin is enriched beneath the cell vertices. The concentration of f-actin at the vertices of the lateral membrane delineates the radial cell column organization of the fiber cells. Bars: (A and B) 5 μm; (C and D) 10 μm.

magnification, the caldesmon immunofluorescence labeled cartwheel-like structures in the BMC, presumably the f-actin bundles observed earlier (compare with Fig. 11B). The caldesmon antibody evenly stained the lateral membranes at the BMC and elsewhere along the fiber length. The N-cadherin/f-actin rich foci in the BMC (Fig. 11B) were not enriched in caldesmon.

In many non-muscle cells, myosin light chain kinase (MLCK) phosphorylates one of the two light chains on each head of a myosin II molecule. Following light chain phosphorylation, the myosin head interacts with an actin filament leading to contraction. The presence of MLCK on western blots of BMC samples (Fig. 9) implied the presence of a myosin substrate and suggested that the BMC may have contractile properties. We visualized the distribution of myosin by immunofluorescence and f-actin by phalloidin staining. En face views of double-labeled preparations revealed the relative organization of these molecules in the BMC. The f-actin and myosin formed a two-dimensional hexagonal lattice cradling the posterior surface of the lens (Fig. 13A). At higher magnification (Fig. 13B and inset) it was apparent that myosin plaques were localized beneath the center of each basal membrane at the point of convergence of actin filament bundles.

DISCUSSION

During differentiation, fiber cells migrate along the inner surface of the lens capsule. The capsule is composed of type IV collagen (Spiro and Fukushi, 1969) and laminin (Parmigiani and McAvoy, 1984), with variable amounts of fibronectin (Parmigiani and McAvoy, 1984), entactin (Cammarata and Spiro, 1985), fibrillin (Wheatley et al., 1995), heparan sulfate proteoglycans (Parthasarathy and Spiro, 1982; Halfter and Schurer, 1994; Schulz et al., 1997) and tenascin (Menko et al., 1998). The capsule also acts as a repository for growth modulators, such as FGF (Schulz et al., 1997). It has been argued that the interaction between fiber cells and the posterior capsule plays an important role in the control of cell differentiation (Menko et al., 1998). This interaction is mediated entirely by the tiny patch of basal membrane at the posterior tip of the fibers. This region, approximately 60 μm^2 in area, represents less than 0.1% of the total plasma membrane surface.

The attachment of fibers to the capsule is mediated by integrins located in the BMC. Two β1 integrins, β1α3 and β1α6, have been described in the lens (Menko and Philip, 1995). The β1α6 integrin is a laminin receptor and its presence

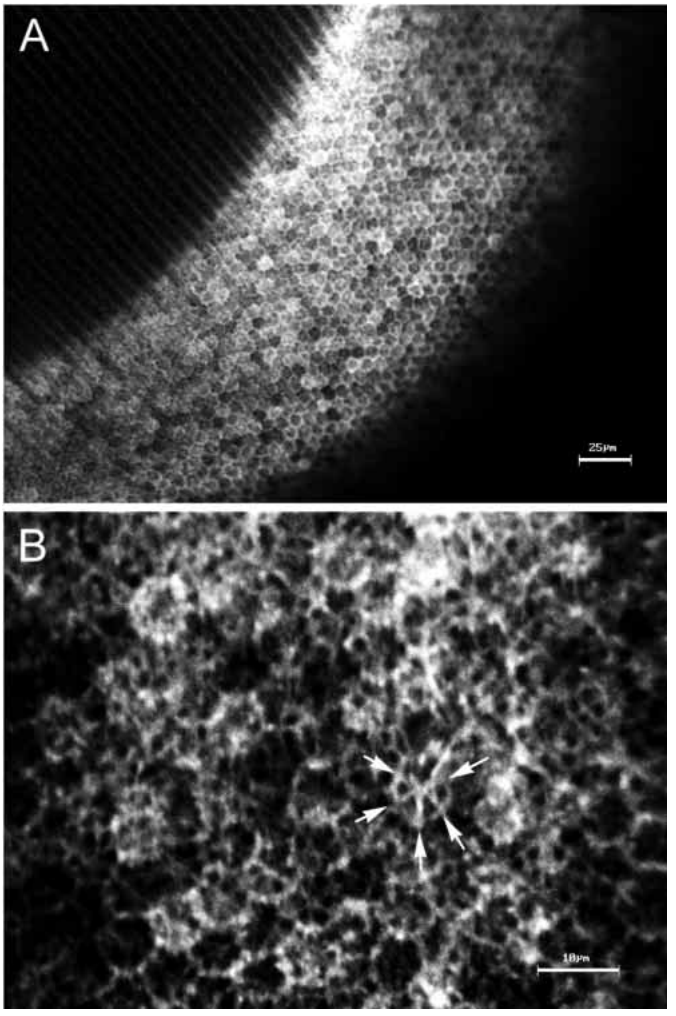

Fig. 12. Organization of caldesmon in the BMC. (A) Oblique optical section through an intact E15 lens showing caldesmon immunofluorescence associated with the lateral membranes and the honeycomb-like BMC. (B) Higher magnification en face view of caldesmon organization in the BMC. Note the hexagonal 'footprints' of the fiber cells on the lens capsule and spoke-like arrangement of caldesmon (arrowed). Bars: (A) 25 μm; (B) 10 μm.

in the BMC, immediately beneath the laminin-rich capsule, is unsurprising. In the present study, we demonstrated a functional role for β1 integrin in the adhesion of fiber cells to the capsule. As noted by others and confirmed here, the distribution of β1 integrin in the lens is not restricted to the BMC (Menko and Philip, 1995; Menko et al., 1998; Walker and Menko, 1998). The β1 integrin is also located in the lateral membranes of fiber cells that are no longer in contact with the capsule. The role of β1 integrin in these cells is unclear, although it may facilitate cell-cell adhesion.

In other epithelial systems, integrin-mediated attachment to the extracellular matrix suppresses apoptosis (Meredith et al., 1993; Frisch and Francis, 1994). Anchorage-dependent cell death, termed anoikis (Frisch and Ruoslahti, 1997), is a feature of many epithelial systems, such as skin (Polakowska et al., 1994) and the gastrointestinal tract (Hall et al., 1994). Lens cells do not undergo anoikis during differentiation. However, a remarkably similar process, that of coordinated organelle loss, occurs shortly after fiber cells detach from the lens capsule. During organelle loss, cytoplasmic organelles are rapidly and synchronously degraded (Bassnett, 1995), ensuring that the optic axis is free of light scattering structures. Organelle loss shares many features of apoptosis including DNA laddering (Appleby and Modak, 1977), appearance of TUNEL-positive nuclei (Bassnett and Mataic, 1997), and cleavage of PARP (Ishizaki et al., 1998). It will be interesting to determine whether components of the BMC (e.g. integrins and FAK) play a role in triggering lens organelle loss and, if so, what the downstream mediators of this process may be.

In some species, fiber cells exceed 1 cm in length. Because the apical and basal membranes of these specialized epithelial cells are so widely separated, fiber cells represent a useful model system in which to study epithelial polarity. In the present work, we developed a novel technique, the capsule stripping procedure, for isolating the fiber cell basal membrane. Previous attempts at biochemical characterization of epithelial cell apical and basolateral domains have relied on complex preparative procedures such as centrifugation through Percoll (Scalera et al., 1980) or sucrose gradients (Kaoutzani et al., 1993) or selective biotinylation of apical or basal surfaces of cultured monolayers (Sargiacomo et al., 1989). The capsule stripping procedure described here provides a basal membrane sample free of contaminating apical membrane and, as judged by the biotinylated membrane protein profiles, demonstrably different from the adjacent lateral membrane. It should be noted, however, that the stripping procedure might not result in the purification of all BMC components. Examination of phalloidin-stained capsules showed that each BMC contained a central plaque of f-actin surrounded by an unstained border region (Fig. 3). The significance of the unstained region is unclear. In undissected lenses, actin is present across the entire inner surface of the capsule (Fig. 11B). The absence of f-actin from regions of stripped capsule may indicate that cytoskeletal components of the BMC contract during isolation, rounding up into a central plaque. Alternatively, the unstained borders may represent regions from which actin (and perhaps other proteins) was lost during stripping. If the latter interpretation is correct, the stripping procedure isolates successfully only the central region of each BMC. Furthermore, although dissection was always performed quickly, readily soluble components of the BMC may be lost during the isolation procedure. This may explain why proteins, such as talin, that are prominent components of focal adhesions, were not identified in the BMC.

The profiles of biotinylated proteins suggested that the BMC differed in composition from the lateral membrane. Moreover, MIP, an abundant lateral membrane protein, was excluded from the basal membrane domain. The maintenance of distinct epithelial apical and basolateral membrane domains is facilitated by the selective delivery of membrane components, and the prevention of lateral mixing of mobile species by tight junctions at the apicolateral margins of the cells. Tight junctions are not known to define a basal membrane domain in the lens or elsewhere. The exclusion of the otherwise ubiquitous MIP could result from its selective insertion into the lateral membrane and immobilization via interaction with lateral membrane proteins or cytoskeletal elements. Alternatively, a previously unrecognized barrier may restrict the diffusion of membrane proteins from the lateral to the basal

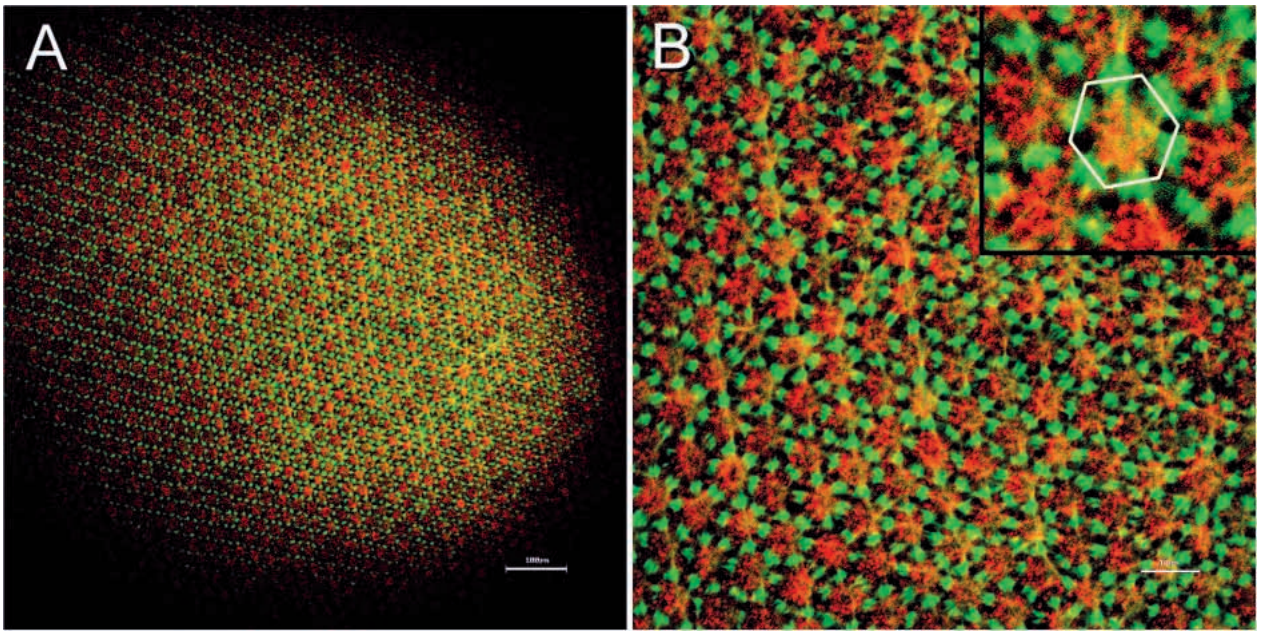

Fig. 13. En face view of the posterior lens face showing the distribution of f-actin (green) and myosin (red). (A) Low magnification view showing the geometric arrangement of f-actin and myosin in the BMC. (B) Myosin is enriched beneath the center of each basal membrane in the region where actin filament bundles converge (see Fig. 10A). The position of the lateral borders of an individual fiber cell is indicated in the inset. Bars: (A) 100 μm; (B) 10 μm.

domain. In neurons, a barrier to the lateral diffusion of lipids and other membrane components is thought to divide the soma-dendritic domain from the axonal domain (Kobayashi et al., 1992).

The arrangement of some adhesive and contractile elements in the BMC is shown in diagrammatic form in Fig. 14. Elsewhere in the lens, f-actin and N-cadherin are concentrated at cell vertices but, in the BMC, these elements are positioned midway along each face of the hexagonal basal membrane. This organization ensures that structural elements in one BMC are aligned with those in a neighboring cell along the three axes of symmetry indicated in Fig. 14. Thus, the posterior lens surface is cradled by a hexagonal latticework of actin filaments. Myosin is located beneath the center of each basal membrane where actin filaments converge. In its organization and composition, the posterior lens surface resembles a two-dimensional muscle.

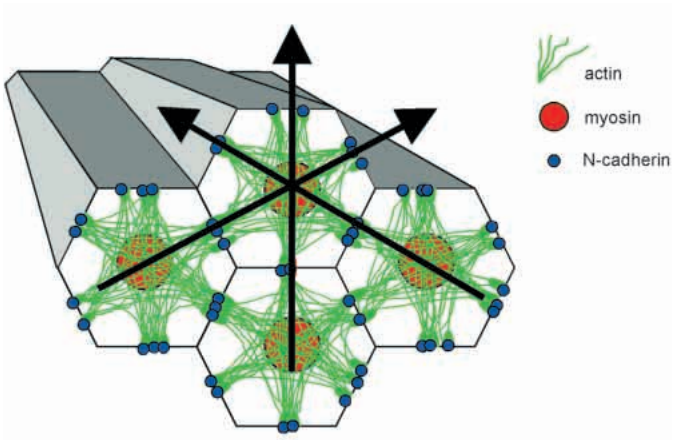

Fig. 14. Arrangement of contractile and adhesive elements in the BMC. The f-actin bundles in the BMC of one cell are aligned with those in the next. Enrichment of N-cadherin at the points of membrane association of actin filaments may stabilize the latticework and ensure that contractile tone generated by actin/myosin interaction is transmitted across the posterior surface of the lens (along the axes indicated by the arrows).

Several functions for the contractile components of the BMC suggest themselves. Actin, myosin, MLCK, and caldesmon are commonly found in migratory epithelial cell types (Nusrat et al., 1992; Saito et al., 1998), so their presence in the BMC may reflect the motile phenotype of differentiating fibers. Alternatively, the generation of contractile tone could play a role in suture formation with the fiber tips being drawn towards the posterior pole by a ‘purse string’ collective contraction of the BMC. However, this mechanism seems unlikely, as none of the axes shown in Fig. 14 are latitudinally oriented. Contraction along a line of latitude would be necessary for a ‘purse string’ mechanism.

If the BMCs generate contractile tone, this would have important implications for the radius of curvature of the posterior lens face. Contraction of the actin/myosin lattice at this surface might play a role in accommodation, a process in which the lens has previously been thought to be entirely passive. This hypothesis could be tested directly by analyzing the accommodative response in the presence of inhibitors of MLCK. Finally, it is possible that the cytoskeletal latticework at the posterior lens surface acts as a sensor rather than as an effector system. Mechanical stresses are important environmental cues for normal and pathological cellular functions, and mechanotransduction shares many of the biochemical characteristics of cell adhesion (such as increased tyrosine phosphorylation of focal adhesions). In several cell

types, integrins serve as the basic cellular mechanotransducers (Shyy and Chien, 1997). At the BMC, actin filaments are linked to integrins that connect the basal membrane of the fiber cells to the overlying elastic lens capsule. This arrangement would seem well suited to monitoring the tensile state of the posterior capsule at rest or during accommodation. By this means, changes in capsule tension could be transduced into appropriate biological responses, such as coordinated growth or differentiation of lens fibers. Interestingly, in human connective tissue disorders, such as Marfan syndrome (Kainulainen et al., 1994) or Weill-Marchesani's syndrome (Jensen et al., 1974), the normal tensile forces exerted on the lens capsule by the ciliary musculature are reduced. In these diseases, the zonules that connect the capsule to the ciliary body become slack. The clinical presentation of patients with these diseases includes high lenticular myopia and spherophakia (the formation of a near spherical lens) suggesting that improper tensioning of the capsule can lead to dramatic changes in the three-dimensional organization of the tissue. If the trophic environment of the eye is unchanged in these conditions, gross abnormalities in the size or shape in the lens may be attributed directly to the absence of normal mechanical cues.

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