Changes in Adhesion Complexes Define Stages in the Differentiation of Lens Fiber Cells

David C. Beebe, Oleg Vasiliev, Jianli Guo, Ying-Bo Shui, and Steven Bassnett

Purpose. During their differentiation, lens fiber cells elongate, detach from the lens capsule, associate at the sutures, and degrade all cytoplasmic membrane-bound organelles. Changes in the expression or organization of cell adhesion and cytoskeleton-associated proteins were correlated with these events during fiber cell differentiation in chicken embryos.

Methods. Fixed or living lenses were sliced with a tissue slicer, permeabilized or extracted with detergents, stained with antibodies or fluorescent-labeled phalloidin, and viewed with a confocal microscope. The distribution of N-cadherin in elongating and mature fiber cells was determined by Western blot analysis. Reverse transcription–polymerase chain reaction (RT-PCR) was used to determine the distribution of vinculin and paxillin transcripts.

Results. Staining for N-cadherin and band 4.1 protein decreased soon after fiber cells detached from the capsule. Detergent extraction of lens sections and Western blots of dissected lens regions showed that much of this decrease in staining was due to epitope masking. Vinculin immunoreactivity was barely detectable on the lateral membranes of elongating fiber cells but increased markedly once these cells reached their maximum length and formed the sutures. Staining for paxillin was also low in elongating fiber cells but increased late in fiber cell differentiation, just before the cells destroyed their membrane-bound organelles. Spectrin and ankyrin immunoreactivity did not change when fiber cells reached the sutures. Staining for F-actin increased transiently in cells that had just reached the sutures. Messenger RNAs for vinculin and paxillin were more abundant in maturing than in elongating fiber cells.

Conclusions. The adhesion complexes of lens fiber cells change in organization and composition soon after these cells finish elongating and detach from the capsule. Increased staining for vinculin and paxillin defines distinct stages of fiber cell differentiation that are intermediate between the completion of cell elongation and the time when lens fiber cells degrade their membrane-bound organelles. Remodeling adhesion complexes during fiber cell maturation may assure the stability of fiber–fiber associations, once these cells are no longer transcriptionally active. (*Invest Ophthalmol Vis Sci.* 2001;42: 727–734)

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Supported by National Institutes of Health Grants EY04853, EY09179 (DCB), and EY09852 (SB), and a National Eye Institute Core Grant to the DOVS. SB is the recipient of a Career Development Award and DCB a Jules and Doris Stein Professorship from Research to Prevent Blindness.

Submitted for publication June 13, 2000; revised November 1, 2000; accepted November 30, 2000.

Commercial relationships policy: N.

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The lens is composed of epithelial cells at various stages of differentiation (Fig. 1A, 1B). The surface of the lens nearest the cornea is covered by a simple cuboidal epithelium. In the avian lens, the epithelium thickens at the lens periphery, forming the annular pad. Mitosis at the border of the epithelium and the annular pad produces cells that will eventually differentiate into the fiber cells that comprise the bulk of the lens. During their differentiation, fiber cells become progressively longer until their tips reach the sutures at the anterior and posterior poles of the lens. At the sutures, they contact fiber cells from the opposite side of the lens (Fig. 1B). Shortly after reaching the sutures, fiber cells detach from the lens capsule and are covered by the next cohort of differentiating cells.[1] Fiber cells deeper in the lens abruptly degrade their nuclei, endoplasmic reticulum, and mitochondria.[2–4] There is a sharp boundary only one or two cells wide between the more superficial fiber cells that contain these organelles and cells deeper within the lens that have no organelles. This boundary defines a stage through which all fibers pass as they mature. Once it is formed early in lens development, the organelle-free zone increases in size at approximately the same rate as the lens. In addition, just before chicken embryo lens fiber cells degrade their organelles, the plasma membranes of adjacent fiber cells partially fuse.[5] This process links the cytoplasm of fiber cells that contain organelles with the cytoplasm of the fiber cells in the organelle-free lens core. Elongating fiber cells also have many fewer membrane interdigitations than mature fiber cells deeper in the lens.[4,6,7]

In equatorial slices, radial cell columns run from deep in the lens to the surface of the fiber mass (Fig. 1A). An elongating fiber cell in one of these columns becomes flattened between the cells that differentiated just before and just after it. Thus, in cross section, a lens fiber cell has two longer sides, where it adheres to the cells in its radial column, and four shorter sides, where it makes contact with cells in the adjacent columns. These cell–cell associations cause the cross sections of fibers to approximate flattened hexagons. The precise packing of the fiber cells may be important for lens transparency, because this order is disrupted in cortical cataracts.

Cell–cell adhesions may stabilize the packing arrangement of lens fibers. They may also be important for holding cells together during accommodation, when substantial bending and shearing forces must be generated between the cells. Several investigators have suggested that the cytoskeleton plays an active or passive role in accommodation.[8–10] To function in this manner, cytoskeletal components must be connected, directly or indirectly, to the junctions that hold fiber cells together.

Previous studies have identified a number of proteins in lens fibers that function as cell−cell adhesion molecules or that serve as links between cytoskeletal elements and the components of cell junctions. One of these is N-cadherin, a transmembrane protein that forms calcium-dependent homophilic associations between neighboring cells.[11–13] Also present in lens fibers are proteins, such as vinculin[14] and plakoglobin,[15] that are likely to connect adhesive junctions to the cytoskeleton. These molecules are most abundant along the shorter sides of elongating fiber cells.[14,15] Several proteins that were first dis-

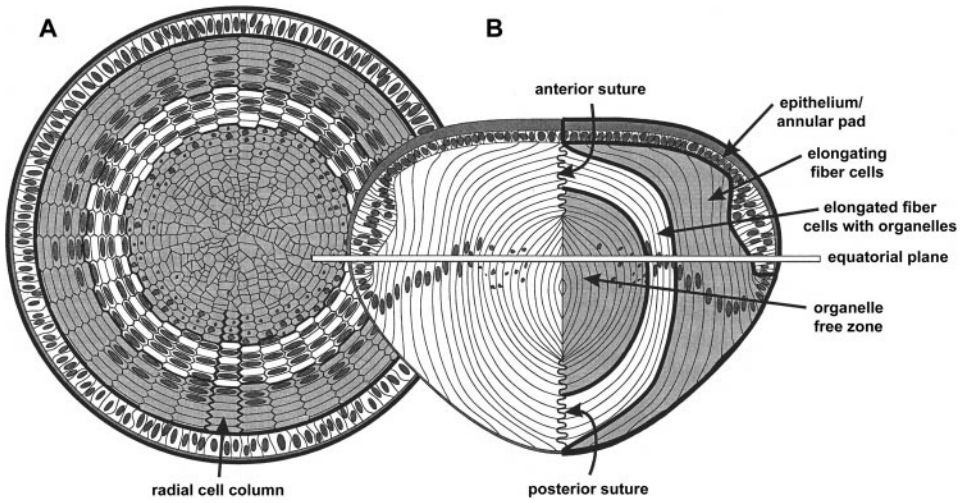

FIGURE 1. Chicken embryo lens sections along the equatorial (**A**) and midsagittal axes (**B**).

covered in the submembrane cytoskeleton of red blood cells have also been identified in the lens. These include spectrin[16–18] (also called fodrin), ankyrin,[19] band 4.1 protein,[16] band 4.9 protein,[20] and band 3 protein.[19] Based on their functions in erythrocytes, these molecules may be important in stabilizing lens fiber cell plasma membranes or in maintaining cell shape. Finally, proteins involved in modulating the assembly, function, and stability of the actin cytoskeleton, including tropomodulin, tropomyosin, caldesmon, and myosin, have recently been described in the lens.[18,21,22]

Actin filaments are known to interact, directly or indirectly, with most of the proteins we have mentioned. Direct association of lens actin with spectrin has been demonstrated.[17] Proteins that link the plasma membrane–adhesion complex with other cytoskeletal elements, such as microtubules and intermediate filaments, have not been identified in the lens, although such links may be important in lens fiber cell formation or stability.

The present study was designed to determine whether proteins that contribute to structure and function of the cytoskeleton–cell adhesion complex change during fiber cell elongation and maturation. We used immunofluorescence to localize several of these proteins in chicken embryo fiber cells. Immunostaining for some proteins changed abruptly during fiber cell maturation. These changes defined two distinct phases of fiber cell differentiation that occurred after fiber cell elongation and before the elimination of intracellular membrane-bound organelles.

METHODS

All procedures used in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. White Leghorn chicken embryos (Truslow Farms, Chestertown, MD) were killed by decapitation between embryonic day (E)6 and E19. In most cases, lenses were removed from the eyes, fixed for 1 to 3 hours in 4% formaldehyde-phosphate-buffered saline (PBS), embedded in 4% agar prepared in 0.9% NaCl or phosphate-buffered saline (0.01 M phosphate buffer [pH 7.4] and 0.15 M NaCl) and sliced at 100 to 400 μm using a tissue slicer (Vibratome Series 1000, TPI, St. Louis, MO; or model OTS 400, Electron Microscopy Sciences, Fort Washington, PA). Slices were cut both parallel and perpendicular to the optical axis to demonstrate fiber cells in longitudinal and transverse section, respectively (Fig. 1). Care was taken that slices passed through or near the center of the lens. Antibody staining was performed in 0.5% Triton X-100 and 10% normal goat serum (Sigma, St. Louis, MO) in PBS for 3 hours to overnight. Slices were washed at least three times with gentle agitation for at least 1 hour with PBS, exposed to secondary antibody in the same buffer used for the primary antibody, washed three times in PBS, and examined with a scanning confocal microscope (model 410; Carl Zeiss, Thornwood, NY).

In some cases, unfixed lens slices (400 or 500 μm) underwent detergent extraction for 3 hours at 4°C in a buffer modified from that described by FitzGerald[23] (0.5% Nonidet P [NP]-40, 100 mM KCl, 5 mM $MgCl_2$, 1 mM disodium EDTA, protease inhibitor cocktail [Roche Molecular Biochemicals, Indianapolis, IN] and 2 mM 2-mercaptoethanol). After extraction, slices were stained as described.

Primary antibodies and the dilutions used were mouse monoclonal anti-N-cadherin (anti-A-CAM, clone GC-4; Sigma), 1:200; rat monoclonal anti-N-cadherin (hybridoma supernatant NC-2), 1:200; mouse monoclonal anti-vinculin (clone hVIN-1; Sigma) 1:200; rabbit anti-ankyrin (chicken erythrocyte; Calbiochem–Novabiochem, La Jolla, CA), 1:200; mouse monoclonal anti-paxillin (clone Z035; Zymed, South San Francisco, CA), 1:200; rabbit anti-α-actinin (Sigma), 1:100; rabbit anti-spectrin (chicken erythrocyte; Sigma), 1:200; mouse monoclonal anti-protein 4.1 (chicken erythrocyte; East-Acres Biologicals, Southbridge, MA), 1:200; and mouse monoclonal anti-talin (Sigma), 1:200. Secondary antibodies were lissamine rhodamine-labeled goat anti-mouse or anti-rabbit IgG or fluorescein-labeled goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), 1:200. Rhodamine phalloidin (1 μg/ml; Sigma) was used to stain actin filaments. In most cases the fluorescent nucleic acid stain (TOTO-1 iodide, 1:10,000; Molecular Probes, Eugene OR) was added along with the secondary antibody to stain the nuclei of the lens fiber cells.

Western blot analysis was performed by standard methods. Cortical (elongating) and nuclear (elongated) fiber cells were dissected from thick lens slices (500 μm) that had been extracted with detergent, as described. Protein concentrations in extracts were determined by a protein assay kit (DC; Bio-Rad, Hercules, CA), using bovine serum albumin as a standard. Extracts were separated on 7.5% polyacrylamide gels. Transfer of similar amounts of protein was confirmed by staining the blots with ponceau S. N-cadherin was detected with the same monoclonal antibody used for immunostaining (1:250), peroxidase-labeled anti-mouse secondary antibody (1:5000) and a luminol chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA).

To determine the relative expression of vinculin and paxillin mRNAs in elongating and maturing fiber cells, freshly dissected E15 lenses were sliced as described earlier, and elongating lens fibers were dissected from fully elongated lens fibers. Total RNA was extracted from these tissues, and reverse transcription–polymerase chain reaction (RT-PCR) was performed on equal amounts of total RNA using standard methods. PCR primers were designed to amplify chicken vinculin and paxillin cDNAs. Two 5′ primers and one 3′ primer were used to generate two vinculin PCR products in separate reactions

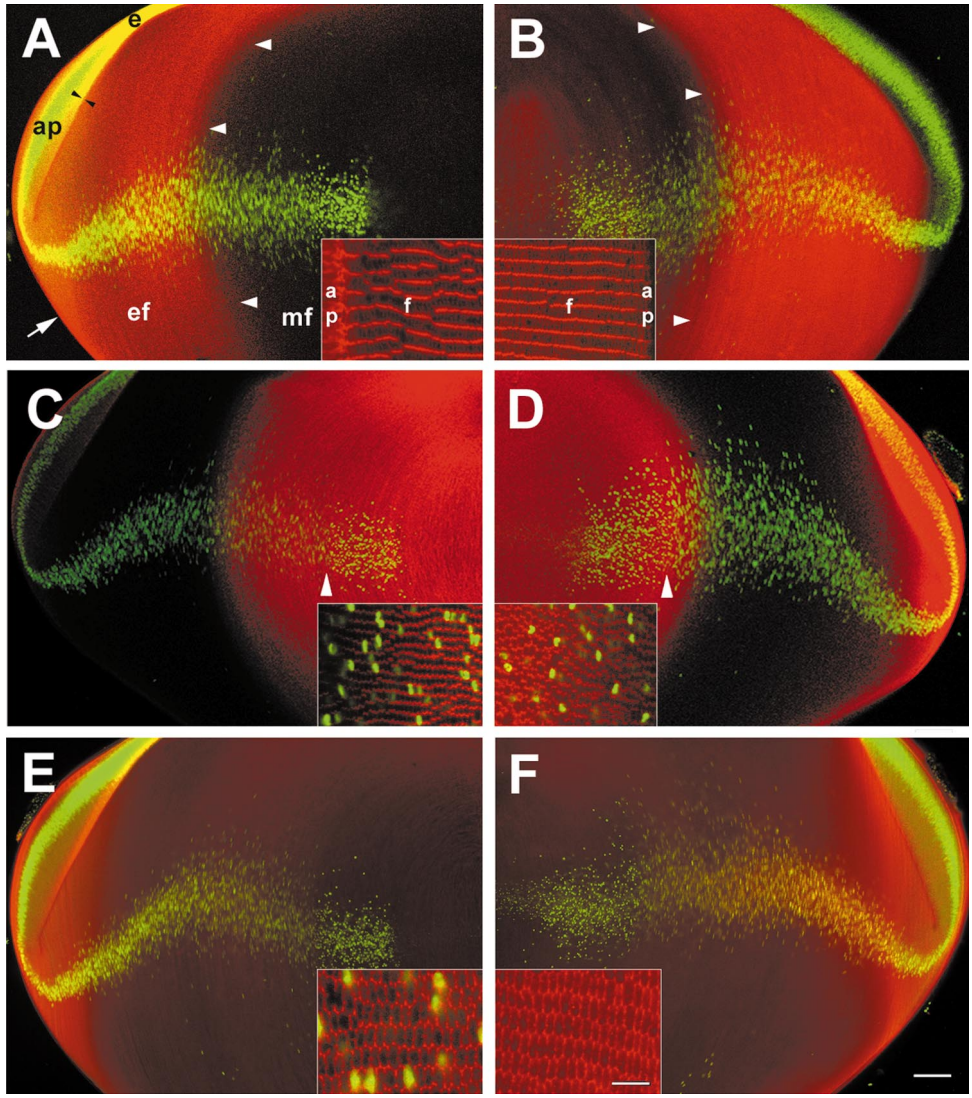

FIGURE 2. The distribution of staining for N-cadherin (**A**), band 4.1 (**B**), vinculin (**C**), paxillin (**D**), ankyrin (**E**), and spectrin (**F**) in midsagittal sections of E17 chicken lenses. *Insets*: Distribution of immunostained proteins in transverse sections of lens fiber cells (immunostained proteins, *red*; nucleic acids, *green*). (**A**) Staining for N-cadherin was seen in epithelial cells (e), annular pad cells (ap), at the apical adherens junctions of annular pad and elongating fiber cells (*black arrowheads*), and in the basal membrane complex (*white arrow*) of elongating fiber cells (ef). Staining declined after elongating fibers reached the sutures (*horizontal white arrowheads*) and became fully elongated, maturing fibers (mf). *Inset*: Staining for N-cadherin was strongest along the short sides of fibers. (**B**) Staining for band 4.1 was not detected in the epithelial or annular pad cells. Elongating fiber cells stained strongly, but staining declined once the elongating fiber cells reached the sutures (*horizontal arrowheads*). Increased staining for band 4.1 was seen in the most central (primary) fiber cells. *Inset*: Staining for band 4.1 was strongest along the short sides of the fiber cells. (**C**, **D**, *vertical arrowheads*) Location of the first condensed fiber cell nuclei, denoting the beginning of the organelle-free zone. (**C**) Immunostaining for vinculin was present in annular pad cells, but was barely detectable along the lateral membranes of elongating fiber cells. Staining for vinculin increased markedly along the lateral membranes of cells that had reached the sutures. *Inset*: Staining for vinculin was strongest along the short sides of the fiber cells. (**D**) Staining for paxillin was strong in the annular pad, decreased in elongating fiber cells, and increased sharply just before the nuclei of the fiber cells were degraded. Staining for paxillin was slightly lower in cells after nuclear condensation. *Inset*: Staining for paxillin was strongest along the short sides of the fiber cells. (**E**) Antibodies to ankyrin strongly stained epithelial cells and cells at the initial stages of fiber cell formation. There was no significant change in staining when fiber cells reached the sutures. Ankyrin immunoreactivity was distributed uniformly on all sides of the fiber cells (*inset*). (**F**) The pattern of staining for spectrin in elongating and maturing fiber cells was similar to that of ankyrin. Spectrin was also detected in all sides of the fiber cells (*inset*). Scale bar, 100 μm (all panels); *insets*, 25 μm.

(forward primer 1, 5′-AAC TGC TAA TAA AAC TAC TGT G-3′; forward primer 2, 5′-CTG TGC AGA CAA CAG AAG ACC AG; reverse primer, 5′ CTG AGG CTG AAA GGC TTC TC). The paxillin primer sequences were: forward 5′-TGC TTT CTC AAA CTC TTC TG-3′ and reverse, 5′-CAC ACA GGC AGA ACC CTA CA-3′. Primers for chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for the amount of cDNA present in each reaction (forward 5′-TCA ATG GGC ACG CCA TCA CT-3′ and reverse 5′-AAG AGC AGG GGC TCC AAC AA-3′). Vinculin and paxillin PCR reactions were run for 29 cycles and GAPDH reactions for 15 cycles (94°C 15 seconds, 56°C 20 seconds, 72°C 2 minutes). PCR products were run on agarose gels and stained with ethidium bromide to reveal the relative abundance of the transcripts.

RESULTS

Distribution of Adhesion-Associated Proteins in the Lens

We first mapped the distribution of N-cadherin (also called A-CAM), a component of cell−cell adherens junctions. Slices cut parallel to the long axis of the fiber cells at E17 (see Fig. 1B for orientation) showed staining for N-cadherin in the cells of the annular pad, in the basal adherens complex at the posterior ends of the elongating fiber cells,[22] and at the apical adherens junctions of the annular pad and elongating fiber cells (Fig. 2A and inset). The lateral membranes of elongating lens fibers were also stained. Cross sections of lens fibers showed that most of the N-cadherin was detected on the short sides of the fiber cells (Fig. 2A, inset). Immunostaining for N-cadherin along the lateral membranes decreased abruptly in deeper fiber cells soon after they detached from the capsule and while they were still nucleated (Fig. 2A). N-cadherin sometimes stained the lateral membranes of primary fiber cells near the center of the lens, although this result was variable (data not shown). The decrease in staining for N-cadherin in cells that had detached from the capsule was similar when two different antibodies to N-cadherin were used (data not shown).

The distribution of band 4.1 protein was also examined during fiber cell maturation. Band 4.1 immunoreactivity was not detected in lens epithelial cells (Fig. 2B). As the fiber cells

began to elongate, staining for band 4.1 appeared along their lateral membranes, most prominently along the short sides of the fibers (Fig. 2B inset). Staining for band 4.1 was not increased in apical or basal adherens junctions (Fig. 2B). However, similar to N-cadherin, band 4.1 immunoreactivity decreased in fiber cells after they detached from the lens capsule.

A monoclonal antibody to vinculin, a protein found in cell−cell and cell–substrate junctions, revealed a pattern of staining in the fiber cells that was, in some ways, reciprocal to that seen with antibodies to N-cadherin (Fig. 2C). Staining for vinculin was present on the basal and lateral membranes of lens epithelial cells and in the apical adherens complexes of epithelial and fiber cells (Figs 2C, 3A). Staining for vinculin was minimal along the lateral membranes of elongating fiber cells, but was prominent at the basal surface of these cells where they attached to the capsule (Fig. 3A). Staining for vinculin at the basal surface of elongating fiber cells decreased as these cells approached the sutures (Fig. 3B). Staining for vinculin increased greatly along the lateral membranes of E17 fiber cells soon after they detached from the capsule (Figs. 2C, 3B). This was the same region of the lens in which staining for N-cadherin decreased. Staining for vinculin was most prominent in the region of the sutures (Fig. 3B) and persisted after the fiber cells degraded their organelles. Similar to N-cadherin and band 4.1 protein, staining for vinculin was strongest along the short sides of the fibers in which this protein was expressed (Fig. 2C, inset).

Immunoreactivity for paxillin, a protein found in focal cell–substrate adhesion plaques, was also found in a distinct distribution along the membranes of lens fiber cells (Fig. 2D). Staining for paxillin was present in the lens epithelial and annular pad cells and at the basal surfaces of elongating lens fibers, but was weak along the lateral membranes of elongating fiber cells. Paxillin immunoreactivity increased abruptly just before the fiber cell nuclei were degraded. The increase in staining for paxillin occurred at approximately the same location where lateral membrane fusions begin to form.[5] Therefore, staining for vinculin and paxillin increased at different stages of fiber cell maturation; vinculin just after fiber cells detached from the capsule and paxillin just before these cells degraded their organelles (Figs. 2C, 2D).

In contrast to the staining patterns described, some proteins appeared to be more uniformly distributed in fiber cells at different stages of maturation. For example, staining for both spectrin and ankyrin was strong in epithelial cells and, after the initial phase of elongation, appeared to be of equal intensity in elongating and maturing fiber cells (Figs. 2E, 2F). In contrast to the other membrane-associated proteins examined, staining for spectrin and ankyrin was uniformly distributed around all lateral fiber cell membranes (insets in Figs. 2E, 2F).

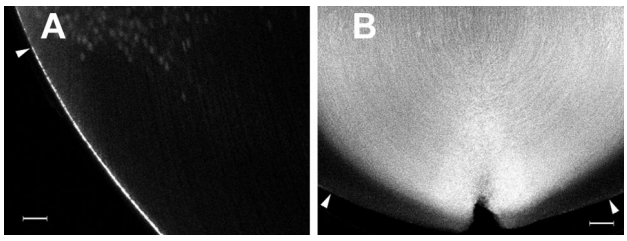

FIGURE 3. Higher magnification images of lenses stained with antibody to vinculin. (**A**) Vinculin stained strongly in the basal ends of elongating fiber cells. Staining increased soon after the fiber cells begin to elongate (*arrowhead*). Faint staining was visible along the lateral membranes of elongating fiber cells. (**B**) Staining for vinculin at the basal ends of the elongating fibers declined as they approached the sutures (*arrowheads*). Staining for vinculin increased markedly along the lateral membranes of fiber cells soon after they reached the sutures and was strongest near the sutures. The separation of the ends of the fiber cells at the posterior suture in this sample was an artifact that occurred during staining. Scale bar, (**A**) 25 μm; (**B**) 100 μm.

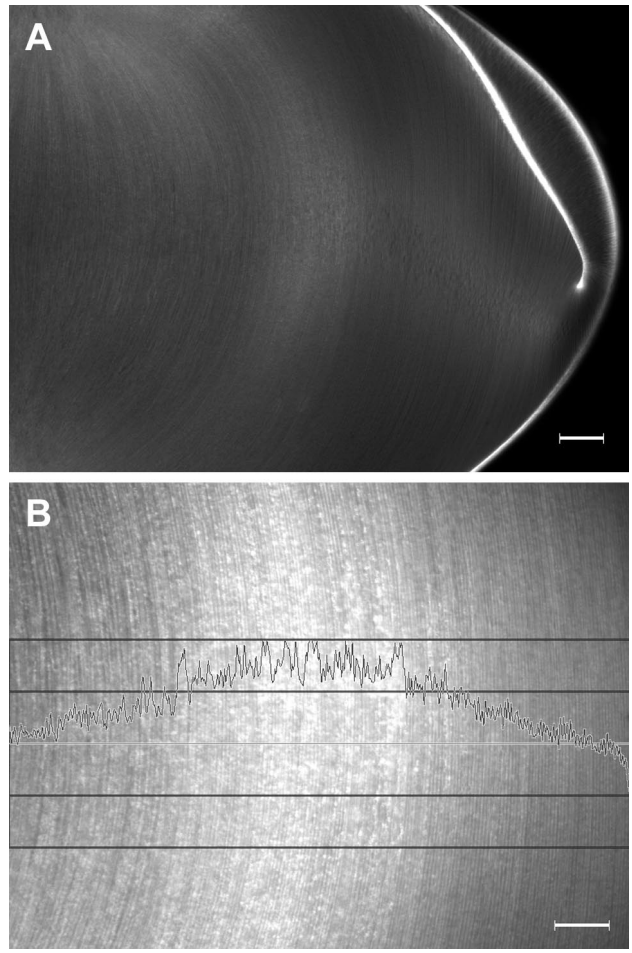

FIGURE 4. Lens sections stained with rhodamine-phalloidin. (**A**) Strong staining was present in the apical and basal adherens complexes of the lens fiber cells. Staining increased along the lateral membranes of the lens fiber cells just after they reached the sutures. (**B**) A higher magnification view of the lateral membranes of fiber cells showing the increase in staining for F-actin at the point where these cells reached the sutures. The graphic overlay shows the extent of change in staining of F-actin in this region. Staining declined again in deeper fiber cells. Scale bar, (**A**) 100 μm; (**B**) 50 μm.

All the molecules examined in this study interact directly or indirectly with the actin cytoskeleton. We, therefore, stained lenses with rhodamine-labeled phalloidin to determine whether the distribution of F-actin changed after fiber cells reached the sutures. Figure 4A shows that strong staining for F-actin was present at the apical and basal ends of the epithelial and elongating fiber cells. Lower levels of staining were seen along the lateral membranes of all fiber cells. A region of increased staining was consistently seen in fiber cells just after they reached the sutures (Figs. 4A, 4B).

Changes in Adhesion Complexes during Early Lens Development

Differences in the spatial distribution of adhesion-associated proteins in fiber cells of E17 lenses could result from one of two different patterns of expression. They could arise because early in lens development fiber cells make one kind of adhesion complex in which paxillin and vinculin predominate. Fiber

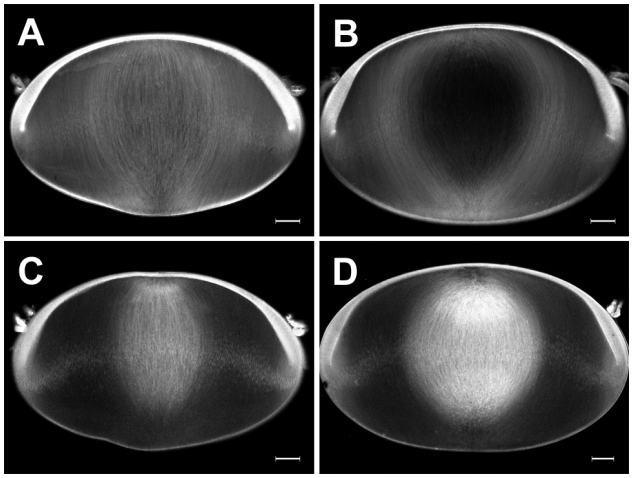

FIGURE 5. The distribution of staining for N-cadherin and vinculin during early stages of lens development. (**A**) Staining for N-cadherin was present in basal and apical adherens complexes and along the lateral membranes of all lens fiber cells at E7. (**B**) By E8 fiber cells in the center of the lens no longer stained for N-cadherin. (**C**) At E7, staining for vinculin was strong in the lens epithelial cells and present at the basal ends of the fiber cells. Staining was weak along the lateral membranes of elongating lens fiber cells. Central fiber cells that had detached from the capsule showed slightly stronger staining. (**D**) By E8 the membranes of central fiber cells stained intensely for vinculin. Scale bar, 100 μm.

cells made later in development would then make adherens complexes containing predominantly N-cadherin and band 4.1 protein. This would cause fibers in the center of E17 lenses, which were produced when the embryo was younger, to have different adhesion proteins than those nearer the surface. Alternatively, no matter at what stage of development they are formed, elongating fibers produce adherens complexes that stain for N-cadherin and band 4.1 protein. When these fibers detach from the capsule they construct junctions that stain predominantly for vinculin. Just before the organelles are degraded fiber cell junctions accumulate increased levels of paxillin.

To distinguish between these possibilities, we immunostained lenses from embryos at E7 and E8. If the first alternative were correct, the fiber cells of these lenses should stain for only vinculin and paxillin. If the second alternative were correct, the youngest lenses should express only N-cadherin and band 4.1 protein in their fibers. Vinculin and paxillin should appear later in the center of the lens in a small region that increases in size as the lens grows.

Figures 5A and 5B show sections through lenses at E7 and E8, respectively, stained with antibodies to N-cadherin. At E7 all fibers stained, whereas at E8 fibers in the center of the lens did not react with the antibody. Similar staining was seen with antibody to band 4.1 protein (data not shown). At E7, light staining for vinculin was present in the central fiber cells that had detached from the capsule (Fig. 5C). By E8, a large area of central fibers showed strong immunostaining for vinculin (Fig. 5D). Staining for paxillin was uniformly very low in the lens fiber membranes at these ages (data not shown). Because the fibers formed early in lens development remain in the center of the lens, the central fibers that did not stain strongly for vinculin at E7 were the same fibers that showed marked staining at E8. Because primary fiber cells first detach from the posterior capsule at E6,[24] increased vinculin expression was seen soon after fiber cells detached from the capsule in E7, E8, and E17 lenses. These data demonstrate that, as lens fiber cells mature, their cell–cell adhesion complexes become different from those found in elongating fibers.

Immunofluorescence of Detergent-Extracted Lens Slices

In contrast to the present results, previous investigators did not observe decreased staining for N-cadherin in deeper fiber cells of the chicken embryo lens (Gerald Grunwald, personal communication, 1995, and Reference 13). However, these earlier studies were performed on frozen sections, and ours used thick sections of formalin-fixed tissue. We hypothesized that, in our studies, the N-cadherin antibodies might have been prevented from interacting with their epitopes in maturing fibers because of reorganization of cell–cell adhesion complexes. In previous studies these epitopes may have been unmasked by the relatively harsh conditions that occur during the preparation and staining of frozen sections.

To test this possibility, lens slices were subjected to detergent extraction before fixation to see whether this treatment would alter the staining patterns in the lens. Lens slices underwent exraction at 4°C with a buffer containing 0.5% NP-40 that was formulated to preserve the lens cytoskeleton and prevent proteolysis.[23] In fixed unextracted lens slices, staining for N-cadherin decreased when fiber cells reached the sutures (Figs. 2A, 6A). After detergent extraction for 3 hours, most of the crystallins were removed from the lens slices, indicating that most cells had been fully permeabilized (Fig. 6B). In immunostained, detergent-extracted lenses N-cadherin immunoreactivity again decreased in fibers after they reached the sutures (Fig. 6C). However, staining for N-cadherin was more evident in the deeper lens fibers of detergent extracted lenses than in lenses

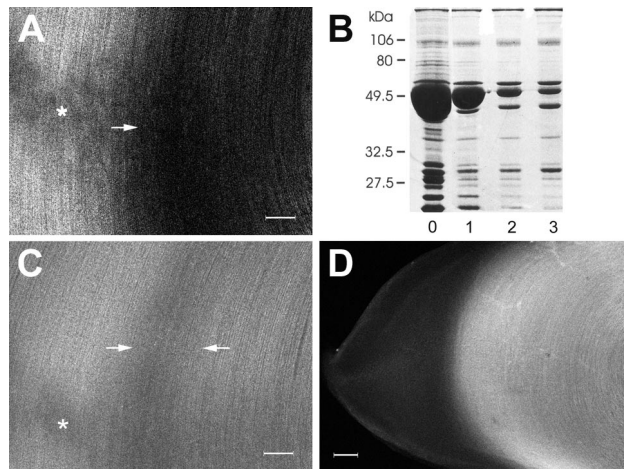

FIGURE 6. Staining for N-cadherin in detergent-extracted lens slices. (**A**) Staining for N-cadherin in a lens section that had not undergone detergent extraction. Staining along the lateral membranes decreased abruptly after the fiber cells reached the sutures (*arrow*). Decreased staining in the region denoted by the *asterisk* was an optical artifact caused by the fiber cell nuclei. (**B**) A Coomassie blue–stained sodium dodecyl sulfate–polyacrylamide gel showing the proteins remaining in lens slices after incubation for varying periods in extraction buffer. The *numbers below the lanes* indicate the number of hours that the slices were subjected to extraction. (**C**) In a detergent-extracted lens slice, staining for N-cadherin again decreased in fiber cells soon after they detached from the capsule (*left arrow*). However, staining of deeper fiber cells was nearly as strong as in elongating fiber cells (*right arrow*). Decreased staining in the region denoted by the *asterisk* was an optical artifact caused by the fiber cell nuclei. (**D**) The distribution of staining for vinculin in lens fiber cells was unchanged after detergent extraction. The lens epithelial and annular pad cells were removed from this specimen before sectioning and detergent extraction. Scale bar, (**A**, **C**) 50 μm; (**D**) 100 μm.

that had not undergone detergent extraction (compare Figs. 6A and 6C). Similar results were obtained for lenses stained with antibodies against band 4.1 protein (data not shown). These observations suggested that the epitopes recognized by antibodies against band 4.1 and N-cadherin were masked in fully elongated lens fibers, compared with elongating fibers. In contrast, the pattern of antibody staining for vinculin (Fig. 6D) and paxillin (not shown) was similar in extracted and unextracted lens slices.

To further explore whether N-cadherin levels are altered after fiber cells detach from the capsule, we dissected detergent-extracted lens slices into cortical (elongating) and nuclear (elongated) fiber cells and assayed for N-cadherin levels by Western blot analysis. When similar levels of total protein were blotted, nuclear fiber cells had less N-cadherin than cortical fiber cells (Fig. 7). This result was consistent with the observation that N-cadherin immunoreactivity decreased slightly along the lateral membranes of fiber cells in detergent-extracted sections after these cells reached the sutures. Decreased levels of N-cadherin in fully elongated fiber cells could also result from the disassembly of the apical and basal adherens complexes that occurs when fiber cells reach the sutures.

Analysis of the Distribution of Vinculin and Paxillin Transcripts by RT-PCR

Freshly isolated E15 lenses were sectioned at 0.5 mm along the optical axis and elongating fibers were separated from fibers that had reached the sutures (see Fig. 1B). Total RNA was extracted from these regions, reverse transcribed, and amplified using PCR primers designed from the sequences of chicken vinculin and paxillin. Primers specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls in the PCR reactions. Figures 8A and 8B provide estimates of the relative abundance of vinculin and paxillin sequences, respectively. In both cases, transcripts were more abundant in maturing fiber cells that had reached the sutures than in elongating lens fiber cells.

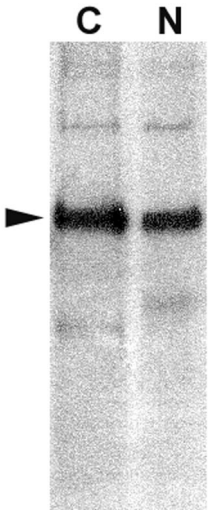

Figure 7. Western blot for N-cadherin showing moderately lower levels of the protein in mature, nuclear (N) compared with elongating, cortical fiber cells (C). Living lens slices underwent detergent extraction, and cortical and nuclear fibers were separated by dissection. Similar amounts of total protein were present on the blots.

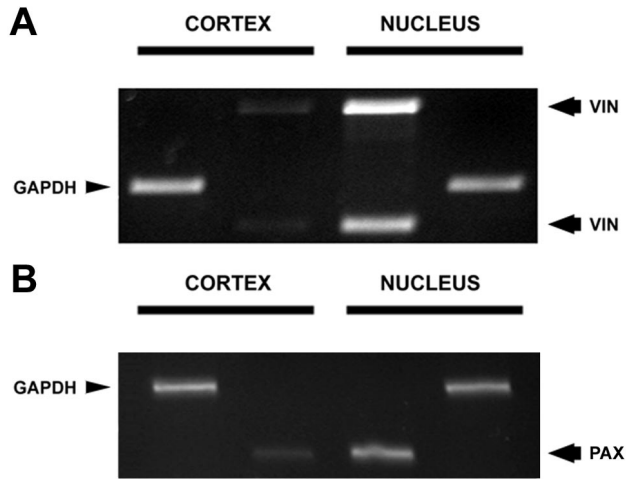

Figure 8. RT-PCR analysis of vinculin and paxillin transcripts in elongating (cortex) and fully elongated (nucleus) lens fiber cells. (**A**) Vinculin PCR products (VIN) were more abundant in the nucleus than in the cortex. The two vinculin PCR products were generated in separate PCR reactions and run on the same gel. Similar amounts of GAPDH PCR products were detected in the two samples. (**B**) RNA from the cortex contained lower levels of paxillin PCR products (PAX) than were amplified from the same amount of total RNA extracted from the nucleus. Levels of GAPDH PCR products were similar in the two samples.

Discussion

The Stages of Fiber Cell Differentiation

The lens grows by the steady addition of fiber cells at its periphery. All fiber cells are retained within the lens. Consequently, fiber cells close to surface of the lens are younger than the cells in the lens nucleus. All lens fiber cells function as optical elements and they therefore share a common elongated prismatic shape. Furthermore, communicating junctions allow the lens to behave as a functional syncytium with respect to small molecules. Faced with an apparently homogeneous system, the tendency has been to attribute those properties of the lens that vary as a function of depth to the effects of aging alone.

However, previous studies and the results presented in this article show that discrete changes occur during fiber cell differentiation. In earlier studies we found that nuclei, mitochondria,[2,3] and the endoplasmic reticulum[4] are abruptly degraded late in the process of fiber formation, well after the fiber cells complete elongation. In addition, in chicken embryo lenses, the membranes of adjacent fiber cells fuse just before they degrade their organelles.[5] The present data show that, at earlier stages, soon after the fiber cells finished elongating, cell adhesion complexes were extensively remodeled. This restructuring involved a reduction of N-cadherin and band 4.1 immunoreactivity and a simultaneous increase in staining for vinculin. Later, at approximately the time when fiber cells fused and just before their organelles were degraded, paxillin immunoreactivity increased markedly.

We propose that fiber cells pass through specific programmed stages during their differentiation. Although these stages may not be marked by gross morphologic alterations, they are accompanied by significant changes in the composition of the fiber cell membrane proteins and can be discerned by the presence or absence of characteristic biochemical markers.

Based on previous observations and those reported in this article, the life of a fiber cell can be divided into at least four

distinct stages. Fiber cells first elongate from columnar progenitor cells at the lens equator. During the period of elongation, their lateral membranes appear relatively smooth, there are distinct adherens junctions near their apical and basal ends and along their lateral membranes, and they contact the capsule and the epithelium at their basal and apical surfaces, respectively. When elongation is complete, fiber cells lose contact with the capsule and the epithelium. Distinct basal and apical adherens complexes can no longer be visualized by antibody staining. At the same time fiber cells appear to restructure their lateral membrane complexes. Staining for N-cadherin and band 4.1 decreases and staining for vinculin increases. These changes are accompanied by an increase in the folding of the lateral membranes of the fiber cells.[4] These membrane surfaces eventually become extensively interdigitated, presumably more firmly locking the fibers to their neighbors.[6,7] An increase in paxillin immunoreactivity just before fibers degrade their membrane-bound organelles marks a third phase of fiber cell maturation. At approximately the same stage of maturation, the fiber cell lateral membranes fuse, creating large pores between adjacent cells.[5] This event links the cytoplasm of cells capable of synthesizing mRNA and protein to the cytoplasm of cells that have lost these synthetic capabilities. The final stage in the life of a fiber cell begins with the loss of its intracellular, membrane-bound organelles.

The value of this proposed staging system is that reference to easily identifiable landmarks that are common to all lenses (such as the presence or absence of organelles and whether fibers have reached the sutures) should allow for direct comparisons between the events that characterize fiber cell maturation in the lenses of different species. In embryonic chicken lenses a fiber cell passes through these stages in a matter of days. For example, a cell that enters the elongation stage on E12 matures and loses its organelles by E17. It will be interesting to determine whether the relative timing of the different stages remains constant throughout embryonic and postnatal life.

The Function of N-Cadherin, Band 4.1 Protein, Vinculin, and Paxillin in Fiber Cell Differentiation

Although staining for N-cadherin and band 4.1 protein followed a similar pattern during fiber cell differentiation, these proteins are known to serve distinct functions in other cell types. N-cadherin is a transmembrane protein that links a cell to its neighbors by associating with the extracellular domains of N-cadherin molecules on adjacent cells. The cytoplasmic domains of cadherins are anchored to the cytoskeleton by a complex of proteins that includes α-catenin, β-catenin, $p120^{ctn}$, and vinculin.[25] Based on its distribution, the major function of N-cadherin in lens fiber cells is likely to be holding cells together along their lateral membranes and stabilizing apical and basal cell−cell adherens junctions. The presence of N-cadherin–containing junctions along the length of the lateral cell membranes of lens fiber cells may also be responsible for the small extracellular space between these cells, a factor that is believed to be important for lens transparency.

Band 4.1 protein links one or more transmembrane proteins to the actin cytoskeleton in nearly all cell types.[26] In erythrocytes, where the function of band 4.1 has been studied most thoroughly, band 4.1 connects the transmembrane proteins, band 3 and glycophorin C, to the actin-spectrin complex. Interaction of band 4.1 with calmodulin can alter the association of the actin cytoskeleton with the membrane, thereby affecting cell shape and the elasticity of the cell surface. Therefore, band 4.1 may help to stabilize the submembrane cytoskeleton of lens fiber cells. Band 3 has been identified in lens fiber cells and is likely to be one of the binding partners for band 4.1.[19] It is not known whether band 4.1 interacts with other lens transmembrane proteins. Interestingly, the antibody used in the present study did not detect band 4.1 in lens epithelial cells, suggesting that band 4.1 is not expressed in epithelial cells or that a different isoform of this protein is expressed as epithelial cells differentiate into fiber cells.

The distribution of vinculin and paxillin described in this article suggests that these proteins may be important in several aspects of fiber cell function. Vinculin is a well-known component of cadherin-containing cell−cell and integrin-mediated cell–substrate complexes, where it plays an important role in regulating the interaction of these complexes with the actin cytoskeleton.[27] Vinculin localized to the apical adherens junctions of lens epithelial and fiber cells, the basal ends of fiber cells at the posterior capsule, and in abundance along the lateral membranes of fiber cells after these cells reached the sutures. The localization of vinculin at the basal ends of elongating fiber cells observed in this study is likely to be involved in anchoring these cells to the posterior capsule by integrin-mediated adhesion.[28] However, the strong staining for vinculin along the lateral membranes of fiber cells that had completed elongation was unexpected. Talin, another component of integrin-mediated cell–substratum complexes, was found at the basal ends, but not along the lateral surfaces of fiber cells (data not shown). This observation suggests that vinculin participates in at least three kinds of adhesion complexes in the lens: apical cell−cell adherens junctions, basal cell–substrate adhesions, and extended cell−cell adhesions along the lateral surfaces of mature fiber cells. It is possible that vinculin-containing adhesion complexes along the lateral surfaces of mature lens fiber cells are important in maintaining lens structure during accommodation and aging. Consistent with this view, staining for F-actin increased when lens fiber cells reached the sutures. Increased staining for vinculin and F-actin may also be related to the increase in membrane interdigitations seen in mature fiber cells.[4,6,7]

Paxillin is a multifunctional component of cell–substrate adhesion complexes that binds to several proteins, including vinculin.[29] The observation that staining for paxillin increased along the lateral membranes of fiber cells at approximately the time they were fusing with their neighbors and just before they lost their organelles is intriguing. Additional studies are required to indicate whether paxillin plays a role in either of these processes. The distinct expression patterns of vinculin and paxillin suggests that these proteins play different roles in mature lens fiber cells.

Changes in N-Cadherin and Band 4.1 Immunoreactivity after Detergent Extraction

Detergent extraction largely reversed the decrease in immunostaining for N-cadherin and band 4.1 seen in deeper fiber cells. The antibodies to N-cadherin used in this study were specific for extracellular epitopes. For this reason we originally thought that the decrease in immunostaining might have been due to changes in the extracellular space in fully elongated fiber cells. However, band 4.1 protein is an intracellular protein. Therefore, the decrease in antibody staining for these proteins reflected a more general alteration in their accessibility to antibodies. It was also striking that staining for ankyrin and spectrin did not decrease in a similar manner, which suggests that the masking of N-cadherin and band 4.1 was relatively specific. Additional studies are needed to reveal the biochemical or structural basis for the decrease in staining for N-cadherin and band 4.1 during fiber cell maturation.

The modest decline in N-cadherin detected by Western blot analysis is consistent with that seen in detergent-extracted lens

slices. A decrease in N-cadherin levels was described previously in the mature fibers of adult human lenses.[30]

Regulation of Vinculin and Paxillin Expression during Fiber Cell Maturation

The increase in vinculin and paxillin immunostaining during fiber cell maturation is likely to be related to the increases that we detected in the transcripts for these proteins. The relative increase in these mRNAs may result from increased transcription. However, we cannot exclude the possibility that the rate of transcription of these mRNAs is unaltered but that they accumulate because they are degraded more slowly in fully elongated fiber cells than in elongating fiber cells. Additional studies are required to distinguish between these alternatives. In support of the first possibility, we recently identified two mRNAs that are first transcribed soon after fiber cells detach from the capsule (Vasiliev and Beebe, manuscript in preparation). This finding demonstrates that transcriptional activation can occur after fiber cells have fully elongated.

Acknowledgments

The authors thank Antonio Pereira for expert assistance, Valery Shestopalov and Beverly Faulkner–Jones for helpful comments on the manuscript, and Gerald Grunwald for kindly providing a rat monoclonal N-cadherin antibody for comparative studies.

References

1. Kuszak JR, Bertram BA, Macsai MS, Rae JL. Sutures of the crystalline lens: a review. *Scan Electron Microsc.* 1984:1369–1378.
2. Bassnett S, Beebe DC. Coincident loss of mitochondria and nuclei during lens fiber cell differentiation. *Dev Dyn.* 1992;194:85–93.
3. Bassnett S. Mitochondrial dynamics in differentiating fiber cells of the mammalian lens. *Dev Dyn.* 1992;11:1227–1232.
4. Bassnett S. The fate of the Golgi apparatus and the endoplasmic reticulum during lens fiber cell differentiation. *Invest Ophthalmol Vis Sci.* 1995;36:1793–1803.
5. Shestopalov VI, Bassnett S. Expression of autofluorescent proteins reveals a novel protein permeable pathway between cells in the lens core. *J Cell Sci.* 2000;113:1913–1921.
6. Kuszak J, Alcala J, Maisel H. The surface morphology of embryonic adult chick lens-fiber cells. *Am J Anat.* 1980;159:395–410.
7. Willekens B, Vrensen G. The three-dimensional organization of lens fibers in the rhesus monkey. *Graefes Arch Clin Exp Ophthalmol.* 1982;219:112–120.
8. Rafferty NS, Goosens W. Cytoplasmic filaments in the crystalline lens of various species: functional correlations. *Exp Eye Res.* 1978; 26:117–190.
9. Rafferty N, Scholz D. Comparative study of actin filament patterns in lens epithelial cells: are these determined by the mechanisms of lens accommodation? *Curr Eye Res.* 1989;8:569–579.
10. Ramaekers FCS, Boomkens TR, Bloemendal H. Cytoskeletal and contractile structures in bovine lens cell differentiation. *Exp Cell Res.* 1981;135:454–461.
11. Volk T, Volberg T, Sabanay I, Geiger B. Cleavage of A-CAM by endogenous proteinases in cultured lens cells and in developing chick embryos. *Dev Biol.* 1990;139:314–326.
12. Takeichi M. The cadherins: cell–cell adhesion molecules controlling animal morphogenesis. *Development.* 1988;102:639–55.
13. Lagunowich LA, Grunwald GB. Expression of calcium-dependent cell adhesion during ocular development: a biochemical, histochemical and functional analysis. *Dev Biol.* 1989;135:158–171.
14. Lo W. Adherens junctions in the ocular lens of various species: ultrastructural analysis with an improved fixation. *Cell Tissue Res.* 1988;254:31–40.
15. Franke W, Kapprell H, Cowin P. Plakoglobin is a component of the filamentous subplasmalemmal coat of lens cells. *Eur J Cell Biol.* 1987;43:301–315.
16. Aster JC, Welsh MJ, Brewer GJ, Maisel H. Identification of spectrin and protein 4.1-like proteins in mammalian lens. *Biochem Biophys Res Commun.* 1984;119:726–734.
17. Green J, Maisel H. Lens fodrin binds actin and calmodulin. *Curr Eye Res.* 1984;3:1433–1440.
18. Lee A, Fischer RS, Fowler VM. Stabilization and remodeling of the membrane skeleton during lens fiber cell differentiation and maturation. *Dev Dyn.* 2000;217:257–270.
19. Allen D, Low P, Dola A, Maisel H. Band3 and ankyrin homologues are present in eye lens: evidence for all major erythrocyte membrane components in same non-erythroid cell. *Biochem Biophys Res Commun.* 1987;149:266–275.
20. Faquin WC, Husain A, Hung J, Branton D. An immunoreactive form of erythrocyte protein 4.9 is present in non-erythroid cells. *Eur J Cell Biol.* 1988;46:168–175.
21. Fischer RS, Lee A, Fowler VM. Tropomodulin and tropomyosin mediate lens cell actin cytoskeleton reorganization in vitro. *Invest Ophthalmol Vis Sci.* 2000;41:166–174.
22. Bassnett S, Missey H, Vucemilo I. Molecular architecture of the lens fiber cell basal membrane complex. *J Cell Sci.* 1999;112:2155–2165.
23. FitzGerald P. Methods for the circumvention of problems associated with the study of the ocular lens plasma membrane-cytoskeleton complex. *Curr Eye Res.* 1990;9:1083–1097.
24. Shestopalov VI, Bassnett S. Three-dimensional organization of primary lens fiber cells. *Invest Ophthalmol Vis Sci.* 2000;41:859–863.
25. Gumbiner BM. Regulation of cadherin adhesive activity. *J Cell Biol.* 2000;148:399–404.
26. Hoover KB, Bryant PJ. The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. *Curr Opin Cell Biol.* 2000;12:229–234.
27. Critchley DR. Focal adhesions—the cytoskeletal connection. *Curr Opin Cell Biol.* 2000;12:133–139.
28. Menko S, Philp N, Veneziale B, Walker J. Integrins and development: how might these receptors regulate differentiation of the lens. *Ann N Y Acad Sci.* 1998;842:36–41.
29. Turner CE. Paxillin. *Int J Biochem Cell Biol.* 1998;30:955–959.
30. Atreya P, Barnes J, Katar M, Alcala J, Maisel H. N-cadherin of the human lens. *Curr Eye Res.* 1989;8:947–956.