The C Terminus of Lens Aquaporin 0 Interacts with the Cytoskeletal Proteins Filensin and CP49

Kristie M. Lindsey Rose,1 Robert G. Gourdie,2 Alan R. Prescott,3 Roy A. Quinlan,4 Rosalie K. Crouch,5 and Kevin L. Schey1

PURPOSE. Aquaporin 0 (AQP0), the most abundant membrane protein in the lens, is a water-permeable channel, has a role in fiber cell adhesion, and is essential for fiber cell structure and organization. The purpose of this study was to identify proteins that interact with the C terminus of AQP0, by using a proteomics approach, and thus further elucidate the role of AQP0 in the human lens.

METHODS. AQP0 C-terminal peptides and AQP0 antibody affinity chromatography were used for affinity purification of interacting human lens proteins. Purified proteins were digested with trypsin, analyzed by liquid chromatography (LC)-tandem mass spectrometry and identified after database searching and manual examination of the mass spectral data. Colocalization of AQP0 with filensin and CP49, two proteins identified after mass spectrometric analysis, were examined by immunofocal and immunoelectron microscopy of lens sections.

RESULTS. The proteomics approach used to identify affinity-purified proteins revealed the lens-specific intermediate filament proteins filensin and CP49. With immunofocal microscopy, regions of colocalization of AQP0 with filensin and CP49 at the fiber cell plasma membrane in the lens cortex were defined. Immunoelectron microscopy confirmed that filensin and AQP0 were present in the same membrane compartments.

CONCLUSIONS. These studies suggest a novel interaction between an aquaporin water channel and intermediate filaments, an interaction through which AQP0 may maintain lens fiber cell shape and organization. (Invest Ophthalmol Vis Sci. 2006;47:1562–1570) DOI:10.1167/iovs.05-1313

Aquaporins are ubiquitous integral membrane proteins that play essential roles in normal physiology and in the pathophysiology of many human disorders. Aquaporin-associated diseases, including cataract and nephrogenic diabetes insipidus, correlate to the tissue in which the aquaporins are differentially expressed.1,2 All aquaporins are water-permeable pores, whereas some also transport small molecules such as glycerol.3 Aquaporin 0 (AQP0), also known as major intrinsic protein (MIP), is the founding member of this family of proteins. Unlike other mammalian aquaporins, AQP0 is both highly expressed and specific to one tissue, the ocular lens. AQP0, the most abundant membrane protein in the lens, functions as a water-selective channel4 and is solely expressed in the lens fiber cells.5

Aquaporins have six transmembrane-spanning domains with their N and C termini located intracellularly. Their cytoplasmic C termini are of particular significance, in that this region is the most structurally diverse among them, and several studies have examined the involvement of their C termini in regulation of protein function.6–8 The most studied regulatory mechanism for aquaporins is AQP2 shuttling in the renal collecting duct. Vasopressin-stimulated phosphorylation of the AQP2 C terminus is required for AQP2 shuttling to the apical membrane.7 In addition, a binding site for actin was found in the AQP2 C terminus and is thought to aid in shuttling;8 however, there is no evidence for regulatory trafficking of lens AQP0. Also, regulatory roles have been indicated for calcium in AQP1 function9 and for calcium and calmodulin in AQP210 and AQP0.11,12 High calcium increased the water permeability of AQP0 in fiber cell vesicles,13 whereas the opposite effect was shown with exogenously expressed AQP0 in oocytes.11,12 However, treatment with calmodulin inhibitors reduced the calcium effects in both systems, and a calmodulin binding site has been identified in the C terminus.13 More recently, AQP0 was shown to bind connexin 45.6 early in the developing chick lens14 and γE- and γF-crystallin in the rat lens.15

The lens is an avascular, transparent tissue that transmits light onto the retina and consists of a single-layer epithelium and the fiber cells that form the bulk of the lens. Epithelial cells continually differentiate into fiber cells, resulting in the ordered packing of concentric layers of fibers that vary in age. The center of the lens, the lens nucleus, contains the most mature fibers, and the lens periphery or cortex contains recently differentiated fibers. In addition to their ordered packing, fibers cells adopt a uniform hexagonal shape and develop lateral fiber cell interdigitations arrayed along the fiber length.16,17 These features serve to limit intercellular space and help reduce light scattering. AQP0 is abundant in the lateral fiber membranes which contain these interdigitations,18 and a role for AQP0 in cell adhesion has been proposed.19 AQP0 has also been found localized in gap junctions and in specialized fiber cell junctions.18,20 In addition, aged fiber cells have altered junction organization and abundance,19,21 lack hexagonal shape, and have irregular interdigitations,19,22 suggesting a structural role and an aging effect on this AQP0 function. Mutations and deficiency of AQP0 cause gross disorganization of the lens fibers and result in cataract.2,21,22 Providing further evidence of a structural role for AQP0 in lens development. Also, AQP0 undergoes a host of posttranslational modifications including truncation, deamidation, and phosphorylation. Most of these are age-related and occur at the C terminus.23
To elucidate further the role of AQP0 in the human lens, we sought to identify interactions between human lens proteins and the C terminus of AQP0—interactions that could be altered with age. AQP0 antibodies were used to purify AQP0/protein complexes, and AQP0 C-terminal peptides were used for affinity purifying the interacting proteins. Proteomics analysis of these proteins revealed the affinity purification of the intermediate filament proteins filensin and CP49. Immunofluorescence microscopy defined specific regions of colocalization of AQP0 with filensin and CP49, and immunoelectron microscopy confirmed that AQP0 and filensin are in close proximity at the fiber cell plasma membrane.

**Materials and Methods**

**Membrane and Cytosolic Protein Preparation**

Human lenses were obtained from the National Disease Research Interchange (Philadelphia, PA). Decapsulated lenses were homogenized in 10 mM NaHCO₃, 5 mM EDTA [pH 8.0] and 10 mM NaF and then centrifuged at 88,000g (20 minutes, 4°C) to separate membrane and cytosolic proteins. Membrane pellets were washed with 10 mM NaHCO₃, 5 mM EDTA [pH 8.0] and then with dH₂O. The protocol adhered to the tenets of the Declaration of Helsinki for research involving human tissue.

**AQP0 C-Terminal Peptide Affinity Purification**

Two peptides identical with regions of the human AQP0 C terminus with sequence CTVGEPVLNQT (residues 251-263) and CAKD-VSNQPEVTVGEPLNQT (residues 240-263) were synthesized at the MUSC Proteogenomics facility with N-terminal cysteines added. A peptide identical with AQP0 extracellular loop residues 110-127 with the sequence CPAVRGNLALNTLHPAVSV was also synthesized. The peptides were reduced with a 50-fold molar excess of dithiothreitol (DTT; 1 hour, 37°C) in 50 mM Tris. Each of the peptides, 1.5 mg, was reconstituted in 50 mM Tris and 5 mM EDTA-Na [pH 8.5] and incubated on separate sulfhydryl-reactive affinity columns (Sulfolink; Pierce, Rockford, IL) for 50 minutes at room temperature. The columns were equilibrated with PBS (phosphate-buffered saline; 0.14 M NaCl, 8 mM Na₂HPO₄, 2 mM potassium phosphate, and 0.01 M KCl [pH 7.4]). A cysteine-blocked column was generated by incubating 2 mL of 0.05 M 1-cysteine-HCl on the affinity column for 2 hours. Human lens cytosolic proteins (5 mg) were digested in PBS with a final concentration of 2.5 mg/mL and incubated on each of the columns. Cytosolic proteins from three human lenses, ages 19, 23, and 37, were used for the peptide column purifications. The columns were extensively washed with PBS, and proteins were eluted with 100 mM glycine (pH 2.8). Eluted proteins were concentrated and washed with dH₂O in 10,000 molecular weight cutoff filters (Centricron; Millipore, Billerica, MA).

**AQP0 Antibody Affinity Purification**

Polyclonal antibodies had been generated against bovine AQP0.²⁴ AQP0 and preimmune antibodies were enriched from rabbit serum by incubating the serum with immobilized protein A (Immunopure; Pierce). Bound antibodies were eluted with 100 mM glycine (pH 2.8) and were exchanged into PBS with 5000 molecular weight cutoff filters (Vivaspin; Vivasience, Carlsbad, CA). To generate AQP0 and preimmune antibody columns, AQP0 and preimmune rabbit antibodies were cross-linked to the immobilized protein A according to the manufacturer’s instructions (Pierce). Briefly, antibodies were incubated with the protein A beads and cross-linked with disuccinimidyl suberate (DSS). The antibody-bound protein A beads were washed with 100 mM glycine, to remove excess DSS and uncleaved antibody. Lens membrane proteins from either whole lenses or cortical fiber cells were solubilized in PBS with 1% vol/vol Triton X-100 or 1% wt/vol octylglu}

Aquaporin 0 Interacts with Filensin and CP49

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Coomassie blue or transferred to nitrocellulose. Rat antibodies diluted 1:5000 were used to probe the separated lens fractions to test antibody specificity. Rat antibodies were detected by rabbit anti-rat antibodies coupled to horseradish peroxidase with diaminobenzidine as substrate.

**Immunocytoconfocal Microscopy**

Bovine lenses were obtained from a local abattoir, cut through the center at the equator, and fixed in 2% wt/vol parafomaldehyde (PFA) in PBS. Fixed tissue was embedded in paraffin, and 5-μm sections were cut from the lens region where the lens was halved on the MUSC Histo-Core Facility cryostat. Sections were rehydrated, permeabilized with 0.1% vol/vol Triton X-100, and blocked with 10% normal goat serum. Lens sections were double labeled for AQP0 and filensin for 1.5 hours with either rabbit polyclonal AQP0 antibody24 (1:100 dilution) and rat polyclonal filensin Q1 antibody (1:200 dilution) or with chicken polyclonal AQP0 antibody (AQP02-S, 1:100 dilution; Alpha Diagnostics, San Antonio, TX) and rabbit polyclonal filensin 3241 antibody30 (1:60 dilution) and rat polyclonal filensin Q1 antibody (1:100 dilution) and rat polyclonal filensin Q1 antibody (1:100 dilution) or with chicken polyclonal AQP0 antibody (AQP02-S, 1:100 dilution; Alpha Diagnostics, San Antonio, TX) and rabbit polyclonal filensin 3241 antibody30 (1:60 dilution). Lens sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 antibody31 (1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with either rabbit polyclonal AQP0 antibody24 (1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 2981 antibody31 (1:100 dilution). Sections were double labeled for AQP0 and filensin for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution) and rabbit polyclonal CP49 for 1.5 hours with chicken AQP0 antibody (AQP02-S; 1:100 dilution).

**Immunoelectron Microscopy**

Bovine lenses were removed from fresh eyes and fixed in 4% PFA in PBS for several days. Pieces of lens were then dissected from the cortex and cryoprotected in 20% polyvinyl pyrolidone in 2.3 M sucrose, mounted on microscope stubs and frozen in liquid nitrogen. Ultrathin sections were cut on a microtome (EMFCS; Leica), collected on 2.3 M sucrose and cryoprotected in 20% polyvinyl pyrolidone in 2.3 M sucrose, mounted on microscope stubs and frozen in liquid nitrogen. Ultrathin sections were cut on a microtome (EMFCS; Leica), collected on 2.3 M sucrose and transferred to pioloform/carbon coated grids (Tokuyasu technique). Sections were blocked in ammonium chloride followed by 0.5% wt/vol fish-skin gelatin and labeled with rat filensin Q1 antibody (1:100 dilution) and rabbit AQP0 antibody (AQP02-S, 1:100 dilution) and rabbit AQP0 antibody (AQP02-S, 1:100 dilution) and rabbit anti-rabbit IgG conjugated with colloidal gold AQP0 was localized with 12-nm gold protein A. Between rounds of labeling the sections were fixed in 0.5% vol/vol glutaraldehyde in water and blocked again. Sections were sealed and contrasted with 2% wt/vol uranyl acetate (9:1) for electron microscopy.

**RESULTS**

**AQP0 C-Terminal Peptide Affinity Purification**

To identify proteins that interact with the C terminus of AQP0, synthetic peptides mimicking the AQP0 C-terminal sequences 251-263 and 240-263 were used for affinity purification of proteins from the human lens cytosolic fraction. Negative controls for these experiments included a cysteine-blocked control column and an affinity column with immobilized synthetic peptides mimicking AQP0 extracellular loop residues 110-127. Affinity-purified proteins were identified with a proteomics approach (i.e., by trypsin digestion of eluted proteins followed by RP-HPLC-tandem mass spectrometry). Proteins identified from the AQP0 C-terminal peptide column eluates were compared with those proteins identified from the control columns, to determine those proteins that specifically interact with the AQP0 C-terminal peptides. SEQUEST database searching, manual interpretation, and PeptideProphet analysis were used to identify and confirm the presence of filensin and CP49 peptides in the eluates. PeptideProphet statistical analysis served to evaluate the peptide sequences identified by SEQUEST and

**TABLE 1. A Comparison of the Number of Filensin and CP49 Peptides Identified after Control and AQP0 C-Terminal Peptide Affinity Chromatography**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cysteine-Blocked Control (3)</th>
<th>Loop Control (2)</th>
<th>Control Total (5)</th>
<th>251–263 C-Terminal Peptide (3)</th>
<th>240–263 C-Terminal Peptide (2)</th>
<th>C-Terminal Peptide Total (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filensin</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>14</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>CP49</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

The numbers in parentheses in the headings are the number of experiments.

**TABLE 2. Filensin and CP49 Peptides Observed in Affinity Experiments**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filensin</td>
<td></td>
</tr>
<tr>
<td>5–11</td>
<td>Ab</td>
</tr>
<tr>
<td>12–23</td>
<td>CC</td>
</tr>
<tr>
<td>51–57</td>
<td>Ct1, Ab</td>
</tr>
<tr>
<td>71–77</td>
<td>Ab</td>
</tr>
<tr>
<td>78–90</td>
<td>Ct1, CC, LC, Ab</td>
</tr>
<tr>
<td>99–106</td>
<td>CC</td>
</tr>
<tr>
<td>99–108</td>
<td>Ab</td>
</tr>
<tr>
<td>119–124</td>
<td>Ab</td>
</tr>
<tr>
<td>144–157</td>
<td>Ct1, Ct2, Ab</td>
</tr>
<tr>
<td>147–157</td>
<td>Ct1</td>
</tr>
<tr>
<td>158–175</td>
<td>Ct1</td>
</tr>
<tr>
<td>158–175</td>
<td>Ab</td>
</tr>
<tr>
<td>212–216</td>
<td>Ct1, Ct2</td>
</tr>
<tr>
<td>223–239</td>
<td>Ct1, Ct2, Ab</td>
</tr>
<tr>
<td>255–276</td>
<td>Ct1</td>
</tr>
<tr>
<td>294–308</td>
<td>Ct1</td>
</tr>
<tr>
<td>294–311</td>
<td>Ab</td>
</tr>
<tr>
<td>312–319</td>
<td>Ct1</td>
</tr>
<tr>
<td>363–368</td>
<td>Ct1</td>
</tr>
<tr>
<td>454–469</td>
<td>Ct2, Ab</td>
</tr>
<tr>
<td>545–560</td>
<td>Ct1</td>
</tr>
<tr>
<td>CP49</td>
<td></td>
</tr>
<tr>
<td>77–89</td>
<td>Ct1</td>
</tr>
<tr>
<td>122–137</td>
<td>Ct1, Ct2, Ab</td>
</tr>
<tr>
<td>155–173</td>
<td>Ct1</td>
</tr>
<tr>
<td>174–191</td>
<td>Ct1, Ab</td>
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<tr>
<td>212–239</td>
<td>Ab</td>
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<td>221–239</td>
<td>Ct1, Ab</td>
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<td>313–318</td>
<td>Ct2</td>
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<tr>
<td>376–388</td>
<td>CC</td>
</tr>
<tr>
<td>389–394</td>
<td>Ct2, Ab</td>
</tr>
<tr>
<td>401–411</td>
<td>Ab</td>
</tr>
<tr>
<td>401–415</td>
<td>Ct1, LC</td>
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</table>

Ab, antibody column; CC, cysteine-blocked column control; Ct1, 251–263 AQP0 peptide column; Ct2, 240–263 AQP0 peptide column; LC, loop control column.
provided a statistical confirmation of the correct identification of eluted peptides.

Two lens-specific cytoskeletal proteins, filensin and CP49, were identified as potential interacting partners of AQP0, as evidenced by significantly more filensin and CP49 peptides identified in the eluates from the AQP0 C-terminal peptide columns when compared with control eluates as shown in Table 1. After identification by SEQUEST, confirmation by PeptideProphet, and manual inspection of the five C-terminal peptide column eluates, 19 filensin peptides and 12 CP49 peptides were identified from the AQP0 C-terminal peptide column eluates. Only four filensin and two CP49 peptides were identified from the five control eluates. Table 2 indicates the sequences of the filensin and CP49 peptides observed, indicating sequence coverage of multiple protein domains. While filensin and CP49 were identified as interacting proteins because they had a higher number of identified peptides in the AQP0 C-terminal peptide affinity eluates than did the control eluates, other proteins including αA-, βB- and βA3-crystallins were identified as nonspecific binders by virtue of significant binding to the control columns (data not shown). Although the filensin and CP49 binding to the peptide column could be indirect, due to binding of a multicomponent complex, no proteins besides filensin and CP49 appeared to bind specifically to the AQP0 C-terminal peptide columns.

AQP0 Immunoaffinity Purification

To identify lens proteins in complex with AQP0 in the human lens, we used AQP0 antibodies for affinity purifying AQP0 and associated proteins from human lens lysates. This approach necessitated detergent solubilization of the AQP0-protein complexes from the lens membrane protein pellet, and the type and concentration of detergent were empirically optimized to solubilize the complexes. Subsequently octylglucoside (1%) or Triton X-100 (1%) were used to solubilize whole lens or cortical fiber homogenates for affinity purification with either AQP0 or preimmune antibodies. After elution of the bound proteins, Western blot analysis with the AQP0 antibody was used to confirm the affinity purification of AQP0 (Fig. 1). Detection of AQP0 in the eluates (Fig. 1A, lanes 8–12) from the AQP0 antibody purification is more evident when compared with the eluates (Fig. 1B, lanes 8–12) from the control preimmune antibodies. Note that AQP0 dimers appear between 40 and 45 kDa.

Eluted proteins were again identified with a proteomics approach. Filensin and CP49 were again identified as interacting partners of AQP0. Several peptides, 11 from filensin and 7 from CP49, were identified with SEQUEST and with manual confirmation of the assignment by inspection of MS/MS spectra (summarized in Table 2). Two mass spectra of a filensin and a CP49 peptide (Figs. 2A, 2B) are shown as examples of the raw MS/MS data that were searched against a human database with SEQUEST to make protein identifications. Figure 2 also demonstrates the method in which these spectra are manually interpreted, with the peaks in the spectra labeled corresponding to b and y product ions. To determine the specificity of filensin and CP49 coeluting with AQP0, an additional set of experiments was performed with the AQP0 and preimmune antibody columns. Filensin and CP49, present in the AQP0 antibody eluates, were absent from the control eluates suggesting a specific interaction with AQP0.

Characterization of the Filensin Q1 Antibody

A polyclonal antibody specific to bovine filensin was generated in rat, and antibody specificity was revealed by Western blot analysis of the pellet (Fig. 3, P) and supernatant (Fig. 3, S) fractions from a 250,000g bovine lens extract. A Coomassie-stained gel demonstrated the high protein load on the gel (Fig. 3, Gel) and therefore the specificity of the antibodies for filensin in these fractions. As can be seen in the Western blot (Fig. 3, Blot), the two major immunoreactive bands corresponded to full-length filensin (Fig. 3, large arrow) and the 53-kDa proteolytic fragment derived from filensin (Fig. 3, small arrow). Some minor fragments were also detected. Filensin undergoes extensive proteolysis during lens fiber differentiation, and thus minor bands were expected. It is also interesting to note a significant signal for filensin in the supernatant fraction of this extract, confirming that soluble forms of filensin are present in the supernatant fractions of the lens.

Colocalization of AQP0 with Filensin and CP49 in Lens Sections

To identify regions of AQP0 and filensin expression in lens fiber cells, bovine lens sections containing fiber cells cut in cross-section were double labeled with rabbit AQP0 and rat Q1 filensin antibodies. In cortical lens fiber cells, AQP0 antibody labeling occurred on all sides of the hexagonal fiber membranes (Fig. 4A). Filensin antibody labeling showed a pattern similar to that of AQP0 with fiber cell membrane localization. Filensin was also detected in cell cytoplasm, although it was predominantly localized at the membrane (Fig. 4B). To elucidate the regions of the membrane where the two proteins colocalized, the images of AQP0 and filensin immunofluorescence were merged (Fig. 4C). AQP0 and filensin colocalization was most apparent at the short sides of the hexagonal fiber cell cross sections and at the apical regions where the six-sided membranes meet. The intense colabeling at the short sides and apices may be due to increased membrane density in these particular regions. This same pattern of colocalization between AQP0 and filensin was also demonstrated with chicken AQP0 and rabbit filensin 3241 antibodies (data not shown). Thus, with two sets of antibodies and different lenses for immunofluorescent microscopy, AQP0 and filensin colocalization was evident at the fiber cell membrane.

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933597/)
Cortical fiber cell cross sections were also double labeled with chicken AQP0 and rabbit CP49 antibodies, to examine colocalization of these two proteins. The distribution of CP49 antibody labeling, visualized as green pixels in Figure 5A, was similar to filensin labeling and showed a localization pattern at the membrane and in the cytoplasm with abundant labeling at the membrane. After merging the AQP0 and CP49 immunofluorescence, colocalization was most intense at the short sides and apical regions of the fiber cell cross sections (Fig. 5C) similar to the results seen with AQP0 and filensin double labeling (Fig. 4C).

**AQP0 and Filensin at the Fiber Cell Membrane**

Specific antibodies to AQP0 and filensin were used to investigate their colocalization by immunoelectron microscopy. Filensin and AQP0, visualized with 8- and 12-nm gold particles, respectively, colocalize at the plasma membrane, confirming the results shown with confocal microscopy. Both filensin and AQP0 antibodies label the same plasma membrane compartments of cortical fiber cells (Fig. 6A; large and small arrows). The labeling density shows a highly specific labeling of the sections by the antibodies, and examples of filensin and AQP0 colocalization to the same area of plasma membrane are shown (Figs. 6B, 6D, pairs of large and small arrows), indicating their close proximity at the membranes.

**DISCUSSION**

In this study, AQP0 antibodies and AQP0 C-terminal peptides were used in complementary approaches to affinity purify and
identify lens proteins that interact with AQP0 in the human lens. The results of the two independent affinity-purification approaches revealed specific affinity for the intermediate filament (IF) proteins filensin and CP49. We interpret these results to indicate an interaction between filensin and CP49 with AQP0; however, these results do not indicate whether the interaction is direct or due to a purified complex. No other proteins appeared to bind the AQP0 C-terminal peptide columns specifically. Although the functional role of AQP0 interacting with filensin and CP49 has not been determined, the recently identified interaction between AQP2 and the microfilament actin is proposed to facilitate AQP2 shuttling in the renal collecting duct. In contrast, there is no evidence for an AQP0-shuttling mechanism in the lens.

The two AQP0-associated proteins identified, filensin and CP49, are lens fiber cell–specific members of the IF protein family.33–35 Intermediate filaments are ubiquitous cytoskeletal structures among vertebrate organisms, with three common structural domains: head, rod, and tail.36 However, filensin and CP49 are quite different from other proteins in this family.37–41 Filensin has a shortened central rod domain, whereas CP49 is completely lacking a C-terminal tail domain. Also, filensin and CP49 show divergence in the highly conserved motifs that border the central α-helical domain of all intermediate filaments. These differences most likely contribute to the formation of a unique lens fiber cell–specific structure called the...
beaded filament. Similar to AQP0, filensin-CP49 filaments are found at all stages of differentiation, from the young cortical fiber cells to the mature fibers in the lens nucleus. As with other lens proteins such as AQP0, filensin and CP49 are proteolytically processed during fiber cell maturation. For this reason, the peptides identified from the affinity purifications were noted for their location in the protein sequence (Table 2). Our studies revealed peptide identifications from the head, rod, and tail domains of filensin and from the head and rod domains of CP49. Note that a major 53-kDa filensin fragment is capable of forming filaments, therefore, such a fragment bound to AQP0 could maintain the IF-AQP0 interaction.

The AQP0 C-terminal peptides that were used for affinity purification mimic a region of AQP0 that is severely affected by age-related truncation. In a recent study involving tandem mass spectrometry, AQP0 was analyzed from concentrically dissected layers of fiber cells of different ages. AQP0 truncation at C-terminal residue 259 was identified in the inner cortex, whereas increasing truncation was found in the nucleus with major truncation sites at residues 246, 243, and 259. Because filensin and CP49 were identified after purification with the distal AQP0 C-terminal peptide, truncation at AQP0 residues 246 and 243 could eliminate the interaction. Therefore, we hypothesize that an age-related reduction in the interaction of AQP0 with filensin and CP49 would occur in the aged lens nucleus. Using immunofocal microscopy, colocalization between AQP0 and filensin/CP49 was observed at the fiber cell membranes in the lens cortex, supporting the biochemical data. Immunoelectronic microscopy confirmed the presence of both filensin and AQP0 in the same regions of cortical fiber membranes, supporting the potential for these proteins to interact. Several studies have demonstrated the localization of filensin and CP49 at the fiber cell membranes; however, the present study reveals that an aspect of this localization may be an association with AQP0.

The AQP0 C terminus has also been shown to interact with calmodulin, and this interaction is a proposed regulatory mechanism for the water permeability of AQP0. However, calmodulin was not identified in the affinity purification approaches used in this study. The C-terminal peptides used for purification are more distal to the putative calmodulin binding site. In the AQP0 antibody approach, calcium was not present in the solubilization buffer, and the interaction of AQP0 with calmodulin is calcium dependent (Lindsey Rose KM, unpublished results, 2005).

The functional significance of the interaction of AQP0 with filensin and CP49 is indicated from the phenotypes of the AQP0-, filensin-, and CP49-knockout mice. Lenses from all three knockouts showed development of light scattering or a cataract that worsened with age. In both CP49- and filensin-knockout lenses, the other filament forming protein of the beaded filament was destabilized and essentially resulted in the double knockout of both filensin and CP49. Consequently, these lenses completely lacked beaded filaments. Ultrastructural studies have been performed on the lenses of AQP0- and CP49-knockout animals, and fiber cells were found to have altered shape and morphology. In the CP49-knockout lenses, the fiber cell interdigitations that are typically arrayed along the length of wild-type fibers were absent, and cortical fibers had no semblance of the uniformity in wild-type fibers. Cortical fibers of AQP0-deficient heterozygote lenses were less uniform in shape and size, and the lateral interdigitations were smaller and irregularly arrayed. Homozygous lenses had nonuniform fiber cell shape and organization in the lens cortex, as seen in the CP49-deficient lenses. However, AQP0 is present at the membrane of CP49-deficient fibers.

**Figure 6.** Immunoelectron microscopy of cortical fibers labeled for AQP0 and filensin. Bovine lens sections were labeled with rat filensin Q1 and chicken AQP0 antibodies. Protein A conjugated to 8- and 12-nm gold particles were used to visualize the proteins by immunoelectron microscopy. AQP0 (12 nm gold) and filensin (8 nm gold) both localized to the plasma membrane compartment. Cytoplasmic filensin (A, arrowbeads) were seen in addition to membrane localization (A, B, D, small arrows). There were several examples in which filensin and AQP0 (A–E, large arrow) occupied adjacent space on the plasma membrane, as shown by the close proximity of 8- and 12-nm particles (B, D, pairs of small and large arrows). Frozen-hydrated sections give a negative contrast to the membranes compared with the positive staining present in conventional resin-embedded sections. Scale bar, 200 nm.
Aquaporin 0 Interacts with Filensin and CP49


