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Identification of a direct Aquaporin-0 binding site in the lens-specific cytoskeletal protein filensin

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Abstract

An interaction between the C-terminus of aquaporin-0 (AQP0) and lens beaded filament protein filensin has been reported previously; however, the region of filensin that is involved in the interaction has not been determined. This study is designed to identify the region of filensin that interacts with AQP0. Chemical crosslinking coupled with mass spectrometry was used to identify the site of interaction. The protein complex was crosslinked with zero-length crosslinker: 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC). The crosslinked membrane fraction was digested by trypsin and crosslinked peptides were identified by liquid chromatography-tandem mass spectrometry. A crosslinked peptide between bovine filensin 451–465 (VKGPKEPEPPADLYTK) and bovine AQP0 239–259 (GSRPSESNGQPEVTGEPVELK) was detected. AQP0/filensin crosslinking was not detected in superficial young fiber cells, but increased with fiber cell age in the lens cortex. AQP0/filensin crosslinking and filensin truncation were observed in the same regions of the lens. This crosslinked peptide can be detected in 75 kDa gel band confirming that AQP0/filensin crosslinking can occur between AQP0 and the filensin C-terminal fragment. These results suggest that the AQP0 C-terminus directly interacts with the region of filensin that is adjacent to the major truncation site and the polybasic cluster of residues in the filensin C-terminal tail. This interaction occurs in a specific region of the lens and could only occur between AQP0 and filensin C-terminal fragment *in vivo*. This interaction supports the dual roles of filensin in the lens; roles that could be important during lens development.

Introduction

The ocular lens is a transparent tissue that consists of two types of cells. A monolayer of epithelial cells underlies the anterior portion of the lens capsule and lens fiber cells comprise the bulk of the lens. Once the lens epithelial cells differentiate into lens fiber cells, lens fiber cells initiate expression of lens fiber cell-specific and –abundant proteins including crystallins, connexin 46 and connexin 50, beaded filament proteins (filensin and CP49) and AQP0. AQP0 is the most abundant membrane protein in the lens constituting 50–60% of the plasma membrane protein (Benedetti et al., 1976) and it plays important roles in

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maintenance of lens transparency and homeostasis. AQP0 has been suggested to perform multiple roles in the lens including functioning as a water channel (Chandy et al., 1997, Francis et al., 2000 and Kushmerick et al., 1995) and as a cell adhesion molecule (Dunia et al., 1987, Gonen et al., 2005, Kumari and Varadaraj, 2009, Michea et al., 1994 and Zampighi et al., 1989). Filensin (BFSP1) expression starts in late stage differentiated lens epithelium (FitzGerald et al., 2016). Filensin is highly abundant in lens fiber cells and co-assembles with CP49 (Phakinin, BFSP2) to form lens beaded filaments (BFs) (Carter et al., 1995, Maisel and Perry, 1972, Merdes et al., 1991 and Merdes et al., 1993). Previous studies have shown filensin is necessary for the long-term maintenance of optical clarity (Alizadeh et al, 2003). Some forms of hereditary cataracts in humans have been linked to mutations of filensin (Ramachandran et al, 2007). These data suggest that filensin is critical for maintaining lens optical properties.

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Primary amino-acid sequence analysis shows filensin is homologous to other members of the intermediate filament protein family (Gounari et al., 1993, Merdes et al, 1991 and Remington, 1993). The structure of filensin is predicted to consist of a head domain, a rod domain and a C-terminal tail domain (Gounari et al., 1993). Compared with other IF proteins, filensin is predicted to have a short rod domain and an unusually long tail domain (Gounari et al., 1993). As fiber cell differentiation progresses, filensin has been shown to be extensively processed during lens fiber cell differentiation (FitzGerald, 1988, FitzGerald, 1991, Fleschner, 1998, Masaki and Quinlan, 1997 and Sandilands et al., 1995). At least two major fragments are formed during proteolytic cleavage; one containing the N-terminal domain and the other containing the C-terminal non-alpha-helical tail domain (Sandilands et al., 1995). Previously, we reported that the major proteolytic cleavage site of filensin is D431 in bovine lens and D433 in human lens (Wang et al., 2010). This major truncation site of filensin is located in a highly conserved C-terminal motif, PEDVPDGxxISKAF (Masaki and Quinlan, 1997) that includes a consensus motif (DXXD) for group II caspases (caspase 2, 3 and 7) (Thornberry et al., 1997) and a consensus sequence for N-myristoyl transferase, G-X-X-X-Ser/Thr-Arg/Lys (Resh, 1999). Truncation at D431 results in the formation of two fragments: one contains the head domain, rod domain and a small part of the tail domain; and the other contains the rest of the tail domain and the newly formed N-terminus of the C-terminal fragment is myristoylated (Wang et al., 2010). Caspase cleavage of D433 in human lens and N-myristoyl transferase catalysis of G434 myristoylation was subsequently confirmed (Quinlan et al., 2012). Posttranslational N-myristoylation of the C-terminal fragment together with the cluster of basic residues explained the membrane binding property of the filensin C-terminal fragment (Sandilands et al., 1995 and Wang et al., 2010). N-myristoylation, a process thought to be co-translational, now has been observed to occur posttranslationally in many proteins in apoptotic cells (Martin et al., 2008). The proteolytic cleavage and post-proteolytic N-myristoylation of filensin suggests an important role of filensin C-terminus during fiber cell differentiation, an apoptotic-like event. The well-established function of filensin to coassemble with CP49 to form lens beaded filaments (Carter et al., 1995, Goulielmos et al., 1996a and Goulielmos et al., 1996b). Structural domains of filensin that are involved in beaded filament formation include the N-terminal head domain, the rod domain, and a partial C-terminal domain. The C-terminal region of the tail domain has been suggested to be not essential for polymerization of beaded filaments

(Sandilands et al., 1995 and Goulielmos et al., 1996b). These results suggest that the N-terminal and C-terminal regions of filensin may play different roles in the lens (Masaki and Quinlan, 1997 and Sandilands et al., 1995).

AQP0 has been reported to interact with filensin and CP49 (Lindsey Rose et al., 2006). The interaction between these important lens proteins is predicted to perform important functions that are unique to fiber cell biology; however, it is not clear whether the interaction is direct or indirect. Since filensin and CP49 co-assemble, it is not clear whether AQP0 interacts with one of them or both and the interaction sites have not been identified. In subsequent work, AQP0 was reported to interact with the filensin C-terminal region, but the specific sites involved in the interaction were not identified (Nakazawa et al, 2011). Since different regions of filensin may play different roles, identification of the interaction site is important to further understand the function of this interaction. In this study, we used chemical crosslinking and mass spectrometry techniques and identified a C-terminal region of filensin that directly interacts with the C-terminus of AQP0. In addition, our result together with previous reports about the localization of filensin C-terminus in lens fiber cells (Oka et al., 2008 and Sandilands et al., 1995) suggests the interaction at this site only occurs between AQP0 and truncated C-terminus in the lens fiber cell. The results provide valuable information to further understand the functions of AQP0 and filensin during fiber cell differentiation. The interaction identified in this study demonstrates the importance of membrane-binding properties of filensin C-terminus and further supports the previous suggestion of dual roles of filensin in the lens (Masaki and Quinlan, 1997 and Sandilands et al., 1995).

Materials and Methods

Preparation of different regions of the bovine lens and EDC crosslinking

Frozen one year old or older bovine lenses (PelFreez Biologicals, Rogers, AK) were used. For identifying crosslinked peptides, a bovine lens was decapsulated and the lens cortex was isolated (2–2.5 mm thickness). The tissue was homogenized in homogenizing buffer (25 mM Tris buffer containing 5 mM EDTA, 1 mM PMSF and 150 mM NaCl, pH 8.0) and centrifuged at 100,000 g for 20 min and the supernatant was discarded. The pellets were washed three times with homogenizing buffer to generate water insoluble fraction (WIF). The WIF was washed with PBS once and resuspended in 1 mL PBS (total protein concentration: 2.5 mg/mL) for crosslinking. The remaining sample was discarded. To quantify the level of crosslinking in the different regions of the lens, the lens was decapsulated and different regions of the lens were isolated by vortexing the lens in 1.5 mL of PBS containing 1 mM PMSF for 5 secs. The remaining lens was frozen on dry ice for diameter measurement. The diameter of the lens was measured on dry ice using a caliper before removing the capsule and after each vortexing step. The radius of original lens was defined as r_0 and the remaining lens after each vortexing was defined as r_1 , r_2 , etc. When only the core was left (diameter of about 1 cm), vortexing no longer removed tissue. The outer surface was then removed by rolling on Kimwipe and only the very middle of the core (7–8 mm) was saved for further experiments and its radius was defined as r_f . The normalized lens distances for samples collected from different regions of the lens were defined as

follows: $R1=r1/r0$, $R2=r2/r0$ and the central core was defined as $rf/r0$ and it includes the whole center of the lens. All samples were centrifuged at 33,000g for 10 min and the supernatant was discarded. The remaining pellets were suspended in PBS and the final protein concentration was adjusted to 2.5 mg/mL for all samples. 1.5 mL was used for the sample obtained from the core of the lens and 300 μ L was used for the other samples. Protein concentrations were obtained by measuring absorbance at $\lambda 280$ using a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE) after solubilizing in 2.5% SDS. An aliquot was saved from each sample collected from different regions of the lens to test for filensin truncation. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC) (ThermoFisher Scientific, Rockford, IL) was immediately added to each sample to a final concentration of 5 mM. The samples were incubated at room temperature for 90 min. Excess EDC was removed by centrifuging at 33,000g and the remaining pellets were washed once with 1 mL of 25 mM Tris buffer containing 5 mM EDTA, 1 mM PMSF and 150 mM NaCl, pH 7.4. The samples were further washed twice with 8 M urea. The samples were then suspended in 100 μ L of 8 M urea and reduced by adding TCEP to 10 mM and incubated at 25°C for one hour. Iodoacetamide was then added to a final concentration of 55 mM. The samples were incubated at 25°C in the dark for 45 min. The samples were centrifuged at 33,000 g for 20 min and the pellets were washed twice with 0.1 M sodium hydroxide. The remaining pellets were further washed with water and suspended in 10% ACN balanced with 50 mM Tris buffer, pH 8.0 and 1 μ g trypsin was added to each sample. The digestion was performed at 37 °C for 18 hours. After digestion, the samples were dried in a speedvac and reconstituted in 5% ACN (0.1% FA).

Crosslinked peptide enrichment

To increase the abundance of crosslinked peptides in the sample for initial crosslinked peptide identification, tryptic peptides were fractionated by offline strong cation exchange as described previously (Wang and Schey, 2011). Briefly, the tryptic peptides of the crosslinked membrane fraction were resuspended in buffer A (5 mM KH_2PO_4 , 30% ACN, 0.05% TFA, pH 2.5). The solution was centrifuged at 20,000 g for 15 min and the supernatant was collected and added to strong cation exchange resin (Luna SCX, 5 μ m, 100 Å media) in a spin cup. After 15 min incubation, unbound peptides were removed by centrifugation at 1000 g. The resin was washed by buffer A twice and bound peptides were step-eluted sequentially by 40%, 60% and 100% buffer B (5 mM KH_2PO_4 , 30% ACN, 0.05% formic acid, 350 mM KCl, pH 3.0) balanced with buffer A. Based on previous results (Wang and Schey, 2011), the 60% buffer B eluate contained enriched crosslinked peptides with C-terminus of AQP0. The 60% buffer B eluate was dried in a speedvac and reconstituted in 0.1% formic acid. The peptides were desalted using a C18 Ziptip (Millipore, Billerica, MA) and eluted in 70% ACN (0.1% formic acid). The eluate was dried in a speedvac and reconstituted in 0.1% formic acid for further LC-MS/MS analysis.

Gel electrophoresis and immunoblotting

All SDS-PAGE supplies were from Invitrogen (ThermoFisher Scientific, Carlsbad, CA). For SDS-PAGE, samples were suspended in 10 μ L of 8 M urea. 10 μ L of 5% SDS was added to solubilize the proteins. The sample was then mixed with SDS sample buffer and sample reducing agent and incubated at 50 °C for 10 min. The samples were then loaded onto a 4–

12% NuPAGE Novex Bis-Tris gel and MOPs running buffer was used for separation. After SDS-PAGE, gels were either stained with GelCode™ Blue Safe Protein Stain (ThermoFisher Scientific, Rockford, IL) or gel-separated proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST (25 mM Tris, 0.15 M NaCl, 0.1% Tween-20, pH 7.5) for one hour. Blots were then incubated with the rabbit anti-filensin C-terminus primary antibody (a gift from Dr. Paul FitzGerald, University of California, Davis CA) in TBST overnight at 4°C followed by six TBST washes for 15 min each. The C-terminal filensin antibody was generated in rabbit against bovine filensin peptide E735-K746 in the distal C-terminal region (Sandilands et al., 1995). 1 µL goat anti-rabbit secondary antibody labeled with Dylight 680 (ThermoFisher Scientific, Rockford, IL) was added and incubated for one hour at room temperature followed by six TBST washes for 15 min each. The results were read using an Odyssey™ Infrared Imager (LI-COR Biosciences, Lincoln, NE).

To analyze proteins in the gel, gel bands were excised and destained with three consecutive washes with a 50:50 mixture of 50 mM ammonium bicarbonate and acetonitrile for 10 min. 50 µL 10 mM DTT were added to each sample and the gel bands were incubated at 56 °C for one hour. Liquid in the tube was removed and 50 µL 55 mM iodoacetamide were then added and the gel bands were incubated at room temperature in the dark for 45 min. The gel bands were then washed and dehydrated once with a 25:75 mixture of 50 mM ammonium bicarbonate and acetonitrile and twice with 50: 50 mixture of 50 mM ammonium bicarbonate and acetonitrile for 10 min each. The gel bands were then completely dehydrated with 100% acetonitrile and dried completely in a speedvac. Gel bands were rehydrated in a 10 µL of solution containing 20 ng/µL trypsin (ThermoFisher Scientific, Rockford, IL) in 50 mM ammonium bicarbonate for 15 min. 30 µL of 50 mM ammonium bicarbonate buffer was added to each sample and the samples were incubated at 37 °C for 18 hours. Peptides were extracted using 5% ACN/0.1% TFA twice, 25% ACN/0.1% TFA once, and 50% ACN/0.1% TFA once. The extracts were pooled and dried in a speedvac and reconstituted in 0.1% formic acid for subsequent LC-MS/MS analysis.

LC-ESI/MS/MS

For crosslinked peptide identification, the 60% buffer B eluate from off-line strong cation exchange fractionation was directly separated on a one-dimensional fused silica capillary column (200 mm × 100 µm) packed with Phenomenex Jupiter resin (3 µm mean particle size, 300 Å pore size). One-dimensional liquid chromatography was performed using the following gradient at a flow rate of 0.5 µL/min: 0–10 min: 2% ACN (0.1% formic acid), 10–50 min: 2–35% ACN (0.1% formic acid), 50–60min: 35–90% ACN (0.1% formic acid) balanced with 0.1% formic acid. The eluate was directly infused into an LTQ Orbitrap Classic mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nanoelectrospray source. MS scans were acquired on the LTQ-Orbitrap with a mass resolving power of 60,000. The instrument was operated in a data dependent mode for all the analyses with the top 5 most abundant ions in each MS scan selected for fragmentation in the LTQ. Singly charged [1+] ions were rejected for fragmentation.

To quantify the level of crosslinking, the trypsin digested samples without enrichment were separated by one-dimensional liquid chromatography using the following gradient at a flow rate of 0.5 μ L/min: 0–2 min: 2% ACN (0.1% formic acid), 2–45 min: 2–30% ACN (0.1% formic acid), 45–65min: 30–95% ACN (0.1% formic acid) balanced with 0.1% formic acid. The eluate from the analytical column was directly infused into a Velos Pro linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA). The instrument was operated in a data dependent mode with additional targeted scans for AQP0 peptide S188-R196 and AQP0/Filensin crosslinked peptide. Dynamic exclusion (exclude after 1 spectrum, release after 15 sec, and exclusion list size of 300) was enabled.

Data analysis

The protein database was a concatenated forward and reversed (decoy) Uniprot bovine database (Oct28, 2013). For identifying the crosslinked peptides, the raw data were manually interpreted or converted to MGF files by an in-house developed algorithm, Scansifter, and crosslinked peptides were identified with the help of the software xQuest (Rinner et al., 2008). A mass tolerance of 5 ppm (for LTQ-Orbitrap) for MS data and 0.75 u for MS/MS data was used. For quantifying the level of crosslinking, the resulting raw files were imported into Skyline (MacLean et al., 2010) for peak-picking and quantitation. Ten product ions (5 from filensin peptide and 5 from AQP0 peptide, shown in supplemental table 1) were used for the crosslinked peptide and 12 ions were used for AQP0 peptide S188-R196 (shown in supplemental table 1). The normalized abundance was then calculated as the ratio of the total peak area from all product ions selected to the total peak area from all product ions of the AQP0 peptide S188-R196.

Results

Identification of crosslinked peptides between filensin and AQP0

Identification of low abundance crosslinked peptides in the presence of a high background of uncrosslinked linear peptides is challenging. After trypsin digestion, crosslinked peptides under acidic conditions have at least four positively charged groups; therefore, crosslinked peptides can be partially enriched by strong cation exchange. After off-line strong cation exchange fractionation, the 60% buffer B eluate was analyzed using a high resolution LTQ Orbitrap mass spectrometer. Previously it has been reported that the region of AQP0 that interacts with filensin is the distal C-terminus (Lindsey Rose et al., 2006). With this in mind, manual searches of the data were done to identify crosslinked peptides linked to the C-terminal AQP0 peptide. The bovine AQP0 C-terminal peptide G239-K259 produces intense fragment ions such as b8, y5, y7 and y8 in MS/MS experiments. Searching for tandem mass spectra with these fragment ions yielded one crosslinked peptide between AQP0 G239-R259 and filensin V451-K465. The data were also searched using xQuest software (Rinner et al., 2008), the MassMatrix algorithm (Xu et al., 2008) and a TagRecon blind search (Dasari et al., 2010) to identify other crosslinked peptides. Other identified crosslinked peptides involving the AQP0 C-terminus include peptides crosslinked with ezrin reported previously (Wang and Schey, 2011) and AQP0/AQP0 crosslinked peptides (unpublished). Crosslinking was not detected between the AQP0 C-terminus and other highly abundant lens proteins such as crystallins indicating the specificity of the crosslinking reaction.

The accurate mass measured for the AQP0/filensin crosslinked peptide was 3947.9777 Da which is 2.4 ppm different from the calculated mass of 3947.9873 Da. With the same precursor masses, two tandem mass spectra for the +4 charge precursor can be detected within a small retention time window. These two tandem mass spectra correspond to crosslinking through different residues. One tandem mass spectrum is shown in Figure 1, which indicates crosslinking occurs between AQP0 D246 (formed after deamination of N246) and filensin K455. D246 is involved in crosslinking as evidenced by the observation that $b6a^+$, $y11a^+$, $y13a^+$ ions do not contain the filensin peptide mass, but the $b8a^+$ ion is shifted by the filensin peptide mass. Another tandem mass spectrum shown in Figure 2 indicates crosslinking occurs through AQP0 E250 and filensin K455. Fragment ions that indicate involvement of E250 residue include the $b8a^+$, $b10a^+$, $b16a^+$, $y8a^+$ and $y13a^+$ ions. Characteristic fragment ions (m/z : 585, 771, 872) of the AQP0 C-terminal tryptic peptide (G239-K259) are observed in both MS/MS spectra and strong y -ions (m/z : 452.8, 904.3, 1130.4) corresponding to cleavage of the proline amide bond of filensin V451-K465 were also present.

Quantification of the level of crosslinking in different regions of the lens

Based on the crosslinking results, the region of filensin that interacts with C-terminus of AQP0 is identified. This region is close to the major truncation site of filensin that occurs with lens fiber cell age. The region of filensin that is involved in the interaction with AQP0 can be found in both the full length and its C-terminal fragment. Whether the observed crosslinked peptide comes from crosslinking of AQP0 with full length of filensin or its C-terminal fragment is not known. Filensin exists as the full length protein in the superficial young fiber cells and with fiber cell age truncation occurs to form a C-terminal fragment and an N-terminal fragment. The extent of crosslinking in different lens regions was measured to determine if the crosslinking reaction occurs throughout the lens. Different regions of the lens were obtained with minimum sample handling before EDC crosslinking to maintain the integrity of the protein complex. The extent of crosslinking in the different regions of the lens is shown in Figure 3A. The experiment was repeated three times and the pattern of crosslinking was reproducible. The crosslinking reaction did not occur in the superficial young fiber cells, increased with age, and then decreased in the nucleus. Previous results using a C-terminal filensin antibody showed filensin signal in the cytoplasm in the young fiber cell (Sandilands et al., 1995), which suggests limited direct interaction with membrane protein AQP0; however, after filensin truncation and subsequent lipidation, the C-terminal fragment moves to the cell membrane (Sandilands et al., 1995, Wang et al., 2010). These results and our observations suggest that, *in vivo*, AQP0 may only interact with the truncated C-terminal fragment. To further confirm the alignment of filensin truncation with AQP0/filensin crosslinking, western blotting using a filensin C-terminal antibody was then performed on samples from different regions of a lens (no crosslinking reagent added). The experiment was repeated three times and one representative result is shown in Figure 3B. Filensin truncation (Figure 3B) and AQP0/filensin crosslinking (Figure 3A) consistently begun to appear in the same region of the lens (after normalized lens distance of 0.9). In addition, a western blot of the EDC crosslinked sample using the filensin C-terminal antibody showed a new band at around 75 kDa (Figure 4A, arrow head). This band was not present in the noncrosslinked sample. This gel band was cut, digested by trypsin and

analyzed by LC-MS/MS. The AQP0/filensin crosslinked peptide can be detected in this gel band (Figure 4B). The gel band from the noncrosslinked sample at the same position was also analyzed in the same way. A faint signal can be detected in the control sample, which is clearly different from the crosslinked sample (Figure 4B). This faint signal in the control sample could be due to native crosslinking; a phenomenon that has been seen in the lens (unpublished).

Discussion

Previously, an interaction between the C-terminus of AQP0 and filensin has been reported (Lindsey Rose et al., 2006), but the specific interaction sites were not identified. The results of the current study identify a site on filensin that is involved in this interaction. The crosslinker EDC used in this study is a zero-length crosslinker and it crosslinks two reactive residues in very close proximity which suggests the AQP0/filensin interaction is a direct interaction.

Filensin is a lens-specific cytoskeletal protein. Previous research determined that filensin is proteolytically processed during fiber cell differentiation (FitzGerald, 1988, FitzGerald, 1991, Fleschner, 1998, Masaki and Quinlan, 1997 and Sandilands et al., 1995) and the major truncation site is D431 in bovine lens and D433 in human lens (Wang et al., 2010). After filensin truncation, the newly formed N-terminus is myristoylated and N-myristoylation together with the cluster of basic residues endowed a membrane binding property to the filensin C-terminal fragment (Sandilands et al., 1995, Wang et al., 2010). The region in filensin that interacts with AQP0 is adjacent to the myristoylation site and a polybasic cluster of residues. The observation that AQP0/filensin crosslinking is absent in the superficial young fiber cells and coincided with the start of truncation suggests that the interaction could only occur between AQP0 and truncated filensin C-terminal fragment. The cellular localization of filensin has been studied by different groups and consistent results have been reported (Grey et al., 2009, Oka et al., 2008 and Sandilands et al., 1995). All of these results support the localization of the C-terminus of filensin in the cytoplasm in young fiber cells where filensin is predominantly in full length form. Therefore, the C-terminus of filensin in the full length form is not colocalized with AQP0 *in vivo*. When filensin is truncated and the myristoylated C-terminal fragment moves to the membrane, AQP0 and filensin C-terminal fragment are spatially available to interact. Thus, this interaction is likely initiated at a specific stage of fiber cell differentiation and will likely decrease with additional fiber cell age since the interaction site in AQP0 is located around a major truncation site of AQP0, i.e. N246 (Schey et al., 2000). Further experiments are needed in human lenses to test this prediction.

Filensin has been suggested to have dual roles in the lens (Sandilands et al., 1995 and Masaki and Quinlan, 1997), and the result reported in this paper further supports this suggestion. A well-established role of filensin is to co-assemble with CP49 to form lens fiber cell-specific beaded filaments (Carter et al., 1995 and Merdes et al., 1993) and the C-terminal region of the tail domain is not essential for polymerization of beaded filament (Oka et al., 2008 and Sandilands et al., 1995). Compared with other intermediate filament proteins, filensin has unusually long tail domain (Gounari et al., 1993) and only part of the

tail domain is essential for forming beaded filaments (Carter et al., 1995). Truncation of filensin results in a portion of the tail domain on the N-terminal fragment and part of the tail domain on the C-terminal fragment (Wang et al., 2010). As described above, with fiber cell age, the C-terminal fragment moves to the membrane (Blankenship et al., 2001 and Oka et al., 2008). The interaction of the C-terminal filensin fragment with AQP0 confirmed a predicted new role of filensin in the process of fiber cell differentiation. Post-translational myristoylation of caspase cleaved proteins has been suggested to represent an elegant means developed by the cell to expand the functionality of its encoded proteins (Martin et al., 2011) and post-translational myristoylation has been widely seen in apoptotic cells (Martin et al., 2008). As a posttranslationally myristoylated protein, filensin is expected to play an important role during fiber cell differentiation, an apoptotic-like process. The interaction between AQP0 with the filensin C-terminus could also suggest an essential role for AQP0 during fiber cell differentiation.

It has been well established that AQP0 performs multiple roles in the lens including functioning as a water channel (Kushmerick et al., 1995, Chandy et al., 1997) and as a cell adhesion molecule (Dunia et al., 1987, Gonen et al., 2005, Kumari and Varadaraj, 2009, Michea et al., 1994 and Zampighi et al., 1989). Whether the filensin/AQP0 interaction identified in the current study is related to these known AQP0 functions or not is not known. A recent immunohistochemical imaging study revealed that AQP0 aggregated into plaque-like structures on the broad side of fiber cells in deeper-lying differentiating fiber cells in rat lens that coincided with the redistribution of filensin from the cytoplasm to cell membrane (Grey et al., 2009). An interaction with filensin could drive AQP0 aggregation on the broad side of fiber cells as previously suggested (Grey et al., 2009). Nakazawa et al (2011) reported that an AQP0/filensin C-terminal interaction reduced AQP0 water permeability and this low water permeability could be needed in deep cortex and nucleus where lens fiber cells have a low metabolism. In another report, Sandilands et al. (1995) predicted the retention of the polar C-terminal fragment of filensin at the plasma membrane could play a role in maintaining the optical properties of the lens by regulating hydration. Further study is needed to clearly understand how this interaction affects lens physiology and transparency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The AQP0 C-terminus directly interacts with the filensin C-terminal tail domain
- The AQP0/filensin interaction spatially localized with filensin truncation products
- This interaction suggests multiple functional roles for both AQP0 and filensin

Figure 1.

Tandem mass spectrum of a quadruply charged crosslinked peptide (m/z: 988.3) between AQP0 239-259 (GSRPSESNGQPEVTGEPVELK) and filensin 451-465 (VKGPKPEPEPPADLYTK) through AQP0 D246 and filensin K455.

Annotation of the crosslinked peptides as a- and b- chains is performed according to Schilling et al. (2003) and AQP0 G239-K259 is assigned to the a- chain and filensin peptide is assigned to the b chain. Only the b- and y- ions are labeled. The sequence of the crosslinked peptide is also shown in the figure. Asterisks indicate fragment ions including crosslinked residues and containing mass of the other chain.

Figure 2.

Tandem mass spectrum of a quadruply charged crosslinked peptide (m/z: 988.3) between AQP0 G239-K259 and filensin V451-K465 through AQP0 E250 and filensin K455. Annotation is same as Figure 1.

Figure 3.

The alignment of AQP0/filensin crosslinking with filensin truncation observations. The different regions of the lens were obtained with minimum sample handling before crosslinking to maintain the integrity of the protein complex. The abundance of AQP0/filensin crosslinking was measured in different regions of the lens and the results from three separate experiments (open circle, closed circle and closed square) are plotted (3A). The normalized lens distances were defined as described in the method. Western blotting was also done to test filensin truncation using an aliquot of sample collected from different regions of the lens before EDC crosslinking (3B).

Figure 4.

Detection of AQP0 and filensin C-terminal fragment crosslinking.

Uncrosslinked and EDC crosslinked samples were separated by SDS-PAGE and western blot was performed using a filensin C-terminal antibody. A 75 kDa gel band can be detected in EDC crosslinked sample (arrow head) (4A). The gel band was cut and digested by trypsin and analyzed by LC-MS/MS. Figure 4B plots signal that matches both precursor mass (m/z : 790.4) and one fragment mass (y_{8b}^{2+} , m/z : 452.7) of the crosslinked peptide.