Specific interaction between lens MIP/Aquaporin-0 and two members of the γ-crystallin family

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Purpose: Major Intrinsic Protein (MIP)/Aquaporin 0 is required for lens transparency and is specifically expressed in lens fiber cell membranes. We have demonstrated previously that in the rat lens MIP interacts specifically with γE-crystallin, resulting in its recruitment to the plasma membrane. Our goal was to examine the interaction or lack of interaction between MIP and all members of the γ-crystallin family and to provide evidence for a physiological role these interactions may play in γ-crystallin or MIP function.

Methods: Full length MIP was expressed as untagged, enhanced green fluorescent protein (EGFP) tagged, or myc tagged proteins. Members of the γ-crystallin family were expressed as red fluorescent protein (HcRed) tagged proteins in the rabbit kidney epithelial cell line RK13. Co-localization of tagged proteins was analyzed by confocal fluorescence microscopy.

Results: Confocal fluorescence microscopy demonstrated that γE- and γF-crystallin co-localize specifically with full length MIP in mammalian cells while other γ-crystallins, including γA-, γB-, γC-, γD-, and γS-crystallin do not. As a result of this interaction, either γE- or γF-crystallin was recruited to the plasma membrane from the cytoplasm. MIP does not interact with the Elo mutant of γE-crystallin, which has been linked to a dominant cataract phenotype in mice.

Conclusions: These experiments demonstrate that MIP interacts selectively with γE- and γF-crystallin, and not with other γ-crystallins. This raises the possibility of MIP playing a structural role in the organization of γ-crystallins in rodent lens fibers and/or that γE- and γF-crystallin may have a specific role in MIP function in the rodent lens.

Lens major intrinsic protein (MIP), also known as Aquaporin 0, is the most abundant protein of the ocular lens fiber membrane. It belongs to an ancient family of membrane channel proteins [1-3]. Mutations in the MIP gene have been linked to genetic cataracts in mice and humans, suggesting an important role for MIP in maintaining lens transparency [4-9]. MIP may play a role in reducing the interfiber space, as tightly packed fibers are required for lens transparency and vacuolated fibers are observed in the mouse mutant lenses. Four mouse MIP mutations, including a point mutation at amino acid 51 (A51P, lop), replacement of the last 61 amino acids (amino acids 203 to 263) at the MIP C-terminus by a transposon sequence (Cat Fr), deletion of MIP amino acids 121 to 175 (Hfl) and deletion of amino acids 46 to 49 (CatΔ121) result in autosomal dominant cataracts and MIP trapping in the endoplasmic reticulum without being inserted into the plasma membrane [4-7]. Similarly, two different point mutations in MIP identified in two human cataract families (E134G, T138R) do not integrate into the plasma membrane when expressed in Xenopus oocytes [8,9].

Water channel activity measurements of reconstituted lens membrane vesicles derived from Cat Fr or MIP null mice, showed a marked decrease in water channel activity compared with those from the wild type lens [10,11]. Severe changes in lens fiber structure with dominant phenotype are observed in MIP mutant mice, suggesting there maybe additional functions for MIP in the lens, such as maintenance of fiber structure and arrangement required for optimal focusing of the lens [11,12].

MIP forms pH and Ca²⁺ dependent water channels when expressed in Xenopus oocytes [13,14] and pH dependent voltage dependent channels in mammalian and insect cells [15]. MIP voltage dependent channels in lipid bilayer vesicles are regulated by protein kinase A dependent phosphorylation [16]. MIP may also function as an adhesion molecule and may play a role in gap junction formation [17-21]. MIP localizes to thin junctions of lens fibers, where it appears to be in a closed water pore configuration [22,23]. Both the N-terminus and the C-terminus of MIP are located in the cytoplasm side of the plasma membrane. The MIP C-terminal polypeptide may play an important role in its physiological function as it is cleaved in cataractogenesis and aging [24-28], is serine phosphorylated [29], and may interact with calmodulin [30,31].

In our previous report, we demonstrated that γE-crystallin, a soluble protein that is also specifically expressed in lens fibers, is an MIP binding protein that interacts with the MIP C-terminal domain. We also demonstrated that this specific interaction results in the recruitment of γE-crystallin to the
plasma membrane in mammalian cells expressing MIP [32]. In the current report, we demonstrate that MIP interacts only with γE- and the closely related γF-crystallin, but not with other γ-crystallins. As a result of this interaction, γE- and γF-crystallins are recruited to the plasma membrane from the cytoplasm.

The γ-crystallins are lens fiber specific soluble proteins that also play a critical role in maintaining lens transparency [33]. Various mutations in different members of the γ-crystallin family have been linked to genetic cataracts in both mice and humans [34-45]. Major mutations in γ-crystallins can result in formation of insoluble aggregates whereas point mutations generally lower solubility [46], causing lens opacity. Our study raises the possibility of a functional link between two distinct classes of lens proteins required for lens transparency.

METHODS

Chemicals and reagents: Turbo Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). Polymerase Chain Reaction (PCR) Purification Kit and Plasmid Midi and Maxi Kits were purchased from Qiagen (Valencia, CA). All other chemicals were reagent grade and from standard commercial sources.

Cell lines: The RK13 (rabbit kidney) cell line was obtained from American Type Culture Collection and maintained as monolayer cultures at 37 °C in a 5% CO2/95% air incubator in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS).

Plasmids: The plasmids pEGFP-C2, pEGFP-N2, and pHcRed1-C1 were purchased from Clontech (Palo Alto, CA). All other plasmid DNAs used were propagated in E. coli strain DH5α or DH10B and purified by ion exchange chromatography using Plasmid Midi Kits or Endonuclease-Free Maxi Kits from Qiagen (Valencia, CA).

DNA sequencing: Sequencing of plasmid DNA was performed using CEQ DTCS-Quick Start Kit (Beckman-Coulter, Fullerton, CA) and an automated, Beckman-Coulter CEQ 2000XL DNA Analysis System, according to the manufacturer’s instructions.

Construction of expression vectors: The expression vector for untagged MIP (pCMV-MIP) was constructed as follows. The full length MIP cDNA fragment was cut from the plasmid pmyc-MIP [32] with restriction enzymes BamHI and EcoRI, purified on a 2% (w/v) agarose gel and cloned into the vector pCMVScript (Stratagene) at the BamHI and EcoRI sites. The resulting plasmid is designated as pCMV-MIP. The expression plasmid for enhanced green fluorescent protein (EGFP) tagged MIP fusion protein (pEGFP-MIP) was constructed as described previously [32]. HcRed tagged γE- and γD-crystallin fusion constructs pHcRed-γE-cry and pHcRed-γD-cry were made as described previously [32]. To construct the HcRed/γ-B-cry fusion plasmid pHcRed-γ-B-cry, the full length rat γB-crystallin cDNA was amplified, (using PCR with turbo Pfu DNA polymerase), from pSport-γB-cry clone jd05h10, obtained from a rat whole eye library (unpublished), with the following amplimers: 5′-ATA TGG AGT CAT GGG AAA GAT CAC CTT CTT C-3′ as the forward primer and 5′-ATA TGT CGA CTC AGT AAA AAT CCA TGA CTC TTC-3′ as the reverse primer. After digestion with BamHI and SalI restriction enzymes and purification using a PCR Purification Kit (Qiagen), the PCR product was cloned into pHcRed1-C1 (Clontech) at the BglII and SalI sites. To construct the HcRed/γ-S-cry fusion plasmid pHcRed-γ-S-cry, the full length rat γB-crystallin cDNA was amplified, (using PCR with turbo Pfu DNA polymerase), from pSport-γS-cry clone jd03a11, obtained from a rat whole eye library (unpublished), with the following amplimers: 5′-ATA TGG A TC CA T GTA CA T CTT ACC CCA GGG-3′ as the forward primer and 5′-ATA TGG ATC CAT GGG AAA GAT CAC CTT CTT C-3′ as the reverse primer. After digestion with BamHI and SalI restriction enzymes and purification using a PCR Purification Kit (Qiagen), the PCR product was cloned into pHcRed1-C1 (Clontech) at the BglII and SalI sites. To construct the HcRed/γ-A-cry fusion plasmid pHcRed-γ-A-cry, the full length rat γA-crystallin cDNA (0.5 kb) was cut from the plasmid pAD-γA-cry [32] with BamHI and SalI restriction enzymes and purified on an 1.2% (w/v) agarose gel. This fragment was subsequently cloned into pHcRed1-C1 (Clontech) at the BglII and SalI sites to produce the plasmid pHcRed-γA-cry. To construct the HcRed/γ-C-cry fusion plasmid pHcRed-γ-C-cry, the full length rat γ-C-crystallin cDNA (0.5 kb) was cut from the plasmid pAD-γC-cry [32] with BamHI and SalI restriction enzymes and purified on an 1.2% (w/v) agarose gel. This fragment was subsequently cloned into pHcRed1-C1 (Clontech) at the BglII and SalI sites to produce the plasmid pHcRed-γ-C-cry. To construct the HcRed/γ-F-cry fusion plasmid pHcRed-γ-F-cry, the full length rat γ-F-crystallin cDNA (0.5 kb) was cut from the plasmid pAD-γF-cry [32] with BamHI and SalI restriction enzymes and purified on an 1.2% (w/v) agarose gel. This fragment was subsequently cloned into pHcRed1-C1 (Clontech) at the BglII and SalI sites to produce the plasmid pHcRed-γ-F-cry.

The expression vectors for EGFP tagged mouse γE-crystallin wild type (pEGFP-γE crystallin) and the Elo mutant (pEGFP-γE Elo) were constructed as follows. The γE-crystallin was cloned by RT-PCR using RNA prepared from C57Bl6 mouse lenses. Sequences for the Ampliprimers were as follows: Forward: 5′-CAT ATG GGA AAG ATC ACC TTC-3′; Reverse: 5′-GGA TTC AAT AGA AAT CCA TGA TTC-3′. The primers included convenient NdeI/EcoRI sites flanking the coding sequence of γE-crystallin to allow subcloning from the cloning and sequencing vector pGEMeasy (Promega, Madison, WI). To clone the γE-crystallin Elo mutation a new forward primer was generated and used in conjunction with the reverse primer above. The new forward primer removed base 404 and introduced a unique ClaI site for subsequent identification. Elo forward: 5′-GCC TAC TGG GTC CTC TAT CGA TGC CCA ACT A-3′. This fragment was also cloned into pGEMeasy (Promega) for sequencing. The full length γE-crystallin Elo mutation was generated by digesting the wild type γE-crystallin construct with PvuII and SphI and replacing this fragment. These constructs were then subcloned into pET23 for bacterial expression using the engineered NdeI and EcoRI sites to pET23CryE and pET23 CryEelo. Subcloning into the pEGFP-C1 vector was achieved by excising the NdeI-
expressing untagged MIP and EGFP tagged wild type and
as described below.

proprietary anti-photo bleaching reagent. Cells were kept at 4
Foster City, CA), an aqueous mounting medium containing a

times with PBS and mounted with Gel Mount (BioMeda Corp.,

Diamidino-2-phenylindole Dihydrochloride, hydrate (DAPI;[

rum was added to each dish which was then incubated at 37

37
C before being imaged by confocal fluorescence microscopy

All expression vectors constructed were under the con-
control of a CMV promoter. DNA sequences of the insert and the
junction regions flanking the insert in these plasmids were
verified by DNA sequencing.

Co-transfection of mammalian cells with EGFP tagged
MIP and HcRed tagged γ-crystallins: RK13 cells were grown
about 30-50% confluence in 60 mm tissue culture dishes. A
transfection mixture was prepared by thoroughly mixing 5 µg
of each plasmid (EGFP or HcRed vectors) in 150 µl of serum
free media with 60 µl of Superfect Transfection Reagent
(Qiagen), incubating the resulting mixture for 20 min at room
temperature followed by addition of 1 ml growth medium con-
taining 10% (v/v) heat inactivated fetal bovine serum. The
transfection mixture was then added to each RK13 culture dish
from which the medium had been removed and the dish was
incubated at 37 °C in a CO2 incubator for 1.5 h. Growth me-
dium (4 ml) containing 10% heat inactivated fetal bovine se-
rum was added to each dish which was then incubated at 37
°C in a CO2 incubator for an additional 40 h. After washing
with PBS three times and complete removal of buffer from the
culture dish, cells were fixed by addition of 2 ml of PBS (pH 7.4; Digene, Gaithersburg, MD) containing 4% (w/v)
paraformaldehyde and incubated for 20 min at room tempera-
ture. Cells were washed 3 times with PBS and complete removal of
buffer from the culture dish, cells were fixed by addition of 2
ml of PBS (pH 7.4; Digene) containing 4% (w/v) paraformal-
dehyde and incubated for 20 min at room temperature. Cells
were washed 3 times with PBS and blocked with 2 ml ICC
buffer [0.5% BSA (w/v), 0.2% (v/v) Tween-20, 0.05% (w/v)
sodium azide in PBS, pH 7.4] containing 5% (v/v) normal
goat serum for 1 h at room temperature. After removal of buffer,
the cells were incubated with 1.5 ml ICC buffer containing 20
µg/ml rabbit anti-human MIP (Alpha Diagnostic International,
San Antonio, TX) or rabbit anti-myc antibody (Cell Signaling,
Beverly, MA) for 1 h at room temperature. After being
washed three times with 4 ml cold ICC buffer (10 min each
wash), the cells were incubated for 1 h at room temperature
with 1.5 ml ICC buffer containing 2.6 µg/ml biotin-SP conju-
gated goat anti-rabbit IgG (Jackson ImmunoResearch Labo-
atories, Inc., West Grove, PA). Cells were washed three times
with 4 ml cold ICC buffer (10 min each wash) and incubated
for 1 h at room temperature with 1.5 ICC buffer containing
2.6 µg/ml Cy3 conjugated streptavidin (Jackson ImmunoResearch Laboratories) and 1 µg/ml 4′,6′-Diamidino-
2-phenylindole Dihydrochloride, hydrate (DAPI). Cells were
washed three times with 4 ml cold ICC buffer (10 min each
wash), followed by washing two times with 5 ml of cold PBS.
Cells were mounted in Gel Mount (BioMeda Corp.) and kept
at 4 °C before being imaged by confocal fluorescence micros-
copy as described below.

Confocal fluorescence microscopy of transfected mam-
nalian cells: The stained and mounted cells in 60 mm culture
dishes were imaged using a Leica TCS SP2 Confocal Micro-
scope with a Leica 40x HCX Plan Apo CS 0.85 NA objective
lens. EGFP fluorescence was imaged at 488 nm (excitation)
and 500-540 nm (emission); HcRed fluorescence was imaged at
568 nm (excitation) and 600-650 nm (emission); Cy3 fluores-
cence was imaged at 568 nm (excitation) and 580-650 nm
(emission) and DAPI fluorescence was imaged at 351/364 nm
(excitation) and 400-500 nm (emission). In order to mini-
mize cross talk between channels, fluorochromes were excited
sequentially rather than simultaneously, using the sequential
scan mode of the confocal microscope.

Spatial quantification of fluorescence: The confocal im-
ages obtained were subjected to a procedure called “spatial
quantification” analysis as described by Fan et al. [32] to
demonstrate plasma membrane co-localization of two or more
proteins. This method measures the fluorescence intensity of
individual fluorescence channel (blue, red, and green) along a
path that goes across the plasma membrane. Peak fluorescence
at the vicinity of the plasma membrane for both fluo-
rescence channels is indicative of plasma membrane co-local-
ization of these fluorescence signals.

Bioinformatics methods: Protein sequences of different
γ-crystallin family members were compared using DNAStar
DNA/Protein sequence analysis software (Madison, WI). Ste-
reo representations comparing the C-terminal domain (III and
IV motifs) of human γD-crystallin and rat γE-crystallin (Fig-
ure 1) were produced using the program PyMOL, with sec-
ondary structure assigned with STRIDE [47]. X-ray crystal
coordinates were used from the following PDB files; 1HK0 for human γD-crystallin [43] and 1A5D for rat γE-crystallin [48].

RESULTS
MIP interacts with γE- and γF-crystallins but not with other γ-crystallins: We have previously demonstrated that specific interaction between MIP and γE-crystallin resulted in the recruitment of γE-crystallin to the plasma membrane from the cytoplasm, as revealed by confocal fluorescence microscopy and co-immunoprecipitation analysis [32]. Under different experimental conditions, we observed that co-localization and lack of co-localization between MIP and γE-crystallin as demonstrated by fluorescence microscopy always corresponded with interaction or lack of interaction between the two proteins as demonstrated by co-immunoprecipitation. For this

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Figure 1. Comparison of rat γE-crystallin and human γD-crystallin C-terminal domains. Stereo representations comparing the C-terminal domain (III and IV motifs) of human γD-crystallin (green) and rat γE-crystallin (cyan). Both are views of the surface which is furthest away from the N-terminal domain. A: Ribbon representation, with motif III β-sheets at the bottom of the screen. The c and d β-strands from both motifs III and IV are colored red and labeled, as are the positions of Asp 114 and Asn 138 to orient the sequence. B: Surface renderings of the human γD-crystallin and rat γE-crystallin C-terminal domains, in the same orientation as in A. The carbon atoms are colored green or cyan as in A. Polar atoms are shown as follows; red for oxygen, blue for nitrogen, and orange for sulfur. There is a cleft (resembling a thumb print) in the surface of rat γE-crystallin running from the left side of the domain image to the middle. The dark blue protrusion to the top of this cleft is the side chain of Arg 163 (indicated with a white star) and the equivalent residue is also marked in the human γD-crystallin surface.
reason, in the current study, we used the co-localization assay to demonstrate the interaction between MIP and all members of the γ-crystallin family (including γA-, γB-, γC-, γD-, γE-, γF-, γD-, and γS-crystallin) by confocal fluorescence microscopy of RK13 rabbit kidney epithelial cells co-transfected with tagged MIP and γ-crystallins. Figure 2 shows the mammalian expression vectors for EGFP tagged MIP and HcRed tagged γ-crystallin family members we constructed for this purpose. The co-transfected cells were subjected to confocal fluorescence microscopy and co-localization of MIP with γ-crystallins and the ability of MIP to recruit the γ-crystallins to the plasma membrane were subsequently examined.

We first confirmed the interaction between MIP and γE-crystallin already observed in our previous study [32]. RK13 cells were co-transfected with expression vectors for EGFP tagged MIP (pEGFP-MIP) and HcRed tagged γE crystallin (pHcRed-γE cry; Figure 3). The EGFP fluorescence (green) was concentrated to the plasma membrane, although cytoplasmic expression was also evident. The HcRed fluorescence was also concentrated on the plasma membrane along with cytoplasmic and nuclear expression (Figure 3). Membrane co-localization was confirmed by spatial quantification analysis, which showed that the EGFP and HcRed fluorescence both peaked at the vicinity of the plasma membrane (see the blue dotted line in Figure 3) when each channel of fluorescence was measured along a path crossing the plasma membrane in the merged image. Thus, these results indicated that γE-crystallin co-localized with MIP at the plasma membrane. In the absence of MIP expression, γE-crystallin showed a diffuse expression pattern throughout the cytoplasm and no membrane

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**Figure 2. Construction of expression vectors for MIP, tagged MIP, and tagged γ-crystallin proteins.**

- **A**: Expression vector pCMV-MIP is used to express untagged MIP.
- **B**: pEGFP-MIP was constructed by fusing the full length MIP to the C-terminal end of EGFP.
- **C**: The full length rat γA-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γA-crystallin.
- **D**: The full length rat γB-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γB-crystallin.
- **E**: The full length rat γC-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γC-crystallin.
- **F**: The full length rat γD-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γD-crystallin.
- **G**: The full length rat γE-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γE-crystallin.
- **H**: The full length rat γF-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γF-crystallin.
- **I**: The full length rat γS-crystallin was fused to the C-terminal end of HcRed to produce HcRed tagged γS-crystallin.
- **J**: The full length mouse γE-crystallin was linked to the C-terminal end of EGFP to produce EGFP tagged mouse γE-crystallin.
- **K**: The full length mouse γE-crystallin Elo mutant was fused to the C-terminal end of EGFP to produce EGFP tagged γE-crystallin Elo mutant. All expression vectors are under the control of a CMV promoter. MIP is indicated as a yellow box, γ-Crystallins are indicated as blue boxes. The EGFP tag is indicated as a green box. The HcRed tag is indicated as a red box. The Myc tag is indicated as a grey box.
Figure 3. Interaction of MIP with γ-crystallins in mammalian cells. RK13 cells were co-transfected with EGFP tagged MIP expression plasmid and one of the following HcRed tagged γ-crystallin expression plasmids: pHcRed-γA cry (Figure 2C), pHcRed-γB cry (Figure 2D), pHcRed-γC cry (Figure 2E), pHcRed-γD cry (Figure 2F), pHcRed-γE cry (Figure 2G), pHcRed-γF cry (Figure 2H), or pHcRed-γS cry (Figure 2I). Forty-eight h after transfection, the cells were fixed with a buffer containing 4% paraformaldehyde and counterstained with the nucleus staining dye DAPI (1 µg/ml). The cells were then visualized by confocal fluorescence microscopy. Images or superimposed images from three channels of fluorescence (DAPI, EGFP, HcRed) were obtained (DAPI+EGFP, HcRed, EGFP+HcRed). Spatial quantification was performed along a path across the plasma membrane, indicated by a white line with prominent end points in the EGFP+HcRed images. EGFP fluorescence and HcRed fluorescence was quantified separately and plotted as a function of distance along the path. Blue lines in the spatial quantification graphs indicate the approximate location of the plasma membrane. The scale bars represents 10 µm. Photographs and spatial quantification graphs shown are representative of those obtained from at least six different fields.
localization was evident (see Figure 4J; also see Figure 4M from Fan et al. [32]), indicating that \( \gamma \)E-crystallin was recruited to the plasma membrane as a result of MIP expression.

We next examined the interaction between MIP and \( \gamma \)F-crystallin, a member of the \( \gamma \)-crystallin family that is the most closely related to \( \gamma \)E-crystallin (98% identity in its protein sequence). RK13 cells were co-transfected with pEGFP-MIP and pHcRed-\( \gamma \)F cry. Both EGFP and HcRed fluorescence showed plasma membrane localization (Figure 3). Plasma membrane co-localization was confirmed by spatial quantification along a path that crosses the plasma membrane in the merged image (Figure 3). These results indicated that MIP co-localized with \( \gamma \)F-crystallin, another member of the \( \gamma \)-crystallin family, at the plasma membrane.

Subsequently, we examined the interaction between MIP and the other members of the \( \gamma \)-crystallin family; \( \gamma \)A-, \( \gamma \)B-, \( \gamma \)C-, \( \gamma \)D-, and \( \gamma \)S-crystallin. When RK13 cells were co-transfected with pEGFP-MIP and pHcRed-\( \gamma \)A cry, the EGFP fluorescence (Figure 3) was localized to the plasma membrane and cytoplasmic space as well. However, the HcRed fluores-

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**Figure 4.** The \( \gamma \)E-crystallin Elo mutant does not interact with MIP. RK13 cells were transfected with one of the following combinations of expression plasmids: pCMV-MIP and pEGFP-\( \gamma \)E cry (A-D), pCMV-MIP and pEGFP-\( \gamma \)E cry Elo (E-H), pEGFP-\( \gamma \)E cry (I-L), or pEGFP-\( \gamma \)E cry Elo (M-P). Forty-eight h after transfection, the cells were fixed with a buffer containing 4% paraformaldehyde. After blocking in ICC buffer containing 5% normal goat serum, the cells were incubated first with 20 \( \mu \)g/ml of rabbit anti-human MIP antibody. After washing, the cells were then incubated with biotin-SP conjugated goat anti-rabbit IgG, followed by washing and incubation with Cy3 conjugated streptavidin and 4,6-diamidino-2-phenylindole dihydrochloride, hydrate (DAPI). The cells were washed extensively and visualized by confocal fluorescence microscopy. For details, see the Methods section. Images or merged images from three channels of fluorescence (DAPI, Cy3, EGFP) were obtained: DAPI+Cy3 (A,E,I,M), EGFP (B,F,J,N), and EGFP+Cy3 (C,G,K,O). Spatial quantification (D,H,L,P) was performed along a path across the plasma membrane, indicated by a white line (C,G,K,O). EGFP fluorescence and Cy3 fluorescence was quantified separately and plotted as a function of distance along the path. Blue lines (D,H,L,P) indicated the approximate location of the plasma membrane. The white bars in C, G, K, and O represent 10 \( \mu \)m. Photographs and spatial quantification graphs shown are representatives of those obtained from at least six different fields.
cience was distributed throughout the cytoplasm and the nucleus (i.e., there was no apparent plasma membrane localization of the HcRed fluorescence). Thus, MIP and α-crystallin did not co-localize to the plasma membrane, which was confirmed by spatial quantification analysis (Figure 3). Therefore, these data indicated that MIP does not interact with γA-crystallin in RK13 cells. Very similar results were obtained when cells were co-transfected with pEGFP-MIP together with γB-, γC-, γD-, or γS-crystallin expression vectors (Figure 3), indicating that MIP does not interact with γB-, γC-, or γS-crystallins.

In summary, MIP interacts only with γE- and γF-crystallins but not with other γ-crystallin family members (including γA-, γB-, γC-, or γS-crystallin). As a result of this interaction, γE- and γF-crystallins but not other γ-crystallins were recruited to the plasma membrane from the cytoplasm by MIP.

**Elo mutation in γE-crystallin prevents the recruitment of γE-crystallin to the plasma membrane by MIP:**

Several inherited cataracts in mice are linked to mutations of γ-crystallins [34,36,37,39,41,42,45,49]. In particular the Elo mutant of γE-crystallin causes severe malformation of the developing mouse lens [50,51]. The Elo mutation disrupts the normal localization of γE-crystallin to the cytoplasm of the lens fibers and localizes instead in the cell nucleus [49]. To determine if the mutant protein is capable of interaction with MIP, RK13 cells were co-transfected with pCMV-MIP (expressing untagged MIP) and pEGFP-Elo mutant (expressing EGFP tagged γE Elo mutant, Figure 2K). The untagged MIP was visualized using an anti-MIP antibody, biotinylated secondary antibody and Cy3 conjugated streptavidin in conjunction with confocal fluorescence microscopy.

MIP, as shown by the red Cy3 fluorescence, was mostly concentrated on the plasma membrane (Figure 4A) with minimal cytoplasmic expression. With pEGFP-γE, the green EGFP fluorescence (i.e., γE-crystallin expression) localized to the plasma membrane although cytoplasmic and nuclear expression was also evident (Figure 3). The appearance of yellow color along the plasma membrane in the merged image (Figure 4C) with the spatial quantification analysis (Figure 4D) confirmed that MIP co-localized with wild type γE-crystallin on the plasma membrane and recruited γE-crystallin to the plasma membrane from the cytoplasm. In contrast, in co-transfection of pCMV-MIP (expressing untagged MIP) and pEGFP-Elo mutant (expressing EGFP tagged γE Elo mutant, Figure 2K) γE-crystallin Elo mutant protein showed only cytoplasmic and nuclear localization (Figure 4G). In the merged image (Figure 4G) the Cy3 and EGFP fluorescence were completely separated from each other; no co-localization was evident, which was confirmed by spatial quantification analysis.

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Figure 5. Alignment of amino acid sequences of γ-crystallins. **A:** The amino acid sequences of rat γA-, γB-, γC-, γD-, γE-, γF-, and γS-crystallins were aligned. Greek key motifs I, II, III, and IV are indicated in brackets. Motif III a, b, c, and d β-strands are indicated in red. Asp 114 and Asn 138 positions of γE-crystallin are indicated. **B:** The amino acid sequences of mouse wild type γE-crystallin and the Elo mutant were aligned.
in the absence of MIP expression (Figure 4I-P), both EGFP tagged wild type γE-crystallin (Figure 4J) and the γE-crystallin Elo mutant (Figure 4N) showed only cytoplasmic and nuclear localization, without any plasma membrane localization. These results indicate that MIP recruits γE-crystallin but not the γE-crystallin Elo mutant to the plasma membrane from the cytoplasm.

Differences between γE-/γF-crystallins and other members of the γ-crystallin family: To search for candidate regions of β-crystallin that are responsible for the specific interaction with MIP, the sequences and structures of the mammalian γ-crystallins were compared. The γ-crystallin family arose by successive gene duplications and each protein polypeptide is built up from four similar modified Greek key motifs [33,52,53]. Primary structure alignment showed that γ-crystallins are most divergent in the third Greek key structural motif of the polypeptide [52] (indicated as bracket III in Figure 5).

γE- and γF-crystallins are the most evolutionarily conserved in amino acid sequence and X-ray structure [48] and are the only two γ-crystallins we have found that interact with MIP. Sequence alignment shows that γE- and γF-crystallin amino acids in the loop between β-strands c and d of motif III are identical and different from all the other γ-crystallins [54] (see also Figure 5). This difference in sequence is also reflected in the three dimensional structure. Figure 1 shows the 3D comparison of the C-terminal domains of human γD-crystallin [48] and rat γE-crystallin [43]. Although the overall domain structure is well conserved and the β-sheets of both proteins superimpose, there are obvious structural differences in the c-d loops. In particular the two c-d loops are much more widely separated in γE-crystallin than in the otherwise very similar γD-crystallin. This is apparent in the ribbon diagrams (Figure 1A). In space filling representations of the same region this has the effect of producing a groove or indentation in γE-crystallin that has the potential to form a binding site (Figure 1B).

DISCUSSION

MIP interacts with γE- and γF-crystallins but not with other γ-crystallins: The γA-F-crystallins in mammals are closely related proteins, expressed from a cluster of genes that has arisen through successive duplications. Although the six proteins are very similar in structure, each has characteristic sequence differences that are conserved among mammalian species. Presumably, this reflects specific evolutionarily selected functions for each family member.

We have previously demonstrated that γE-crystallin can interact with the lens major integral membrane protein MIP, resulting in the recruitment of γE-crystallin to the plasma membrane [32]. Here we extend this observation to the other members of the γ-crystallin family in rat. No interaction occurs between MIP and γA-, γB-, γC-, or γD-crystallins or the more distantly related γS-crystallin. However γF-crystallin, which is most similar in sequence to γE-crystallin, does interact with MIP.

The specificity of the interaction between MIP and γE-/γF-crystallin interaction suggests that these two crystallins have a common binding site for MIP not shared with other members of their family. Indeed, the six members of the group provide a set of natural mutants to map the residues required for MIP binding. In fact, as shown in Figure 5, γE- and γF-crystallin have a unique region of sequence identity, located in their C-terminal domains.

γ-crystallins (and also the related β-crystallins) contain four repeated structural motifs (I-IV), each consisting of four β-strands (α-α). These repeated motifs are arranged in pairs to form two domains (I/II and III/IV). Throughout the βγ-crystallin superfamily, the most variable in sequence of the four motifs is motif III in the C-terminal domain. As shown in the alignment, the sequence between strands c and d of motif III is well conserved between γE- and γF-crystallin but quite different in the other γ-crystallins. This is reflected in differences in the X-ray structures of human γD and rat γE-crystallins, as shown in Figure 1. The wide separation of the c-d loops that run across the domain to link the two β-sheets in γE-crystallin creates a noticeable groove in the molecular surface that is not present in γD-crystallin. This is an intriguing candidate for a binding site and might indeed be involved in the interaction with MIP, although this will need further investigation.

There are other reasons to suspect that the MIP interaction site is in the C-terminal domains of γE- and γF-crystallin. In the experiments described here, the γ-crystallins are all tagged at the N-terminus by reporter proteins (HCRed or EGFP) comparable in size to the crystallin itself. This is likely to block possible interactions involving some regions of the N-terminal domain, but is less likely to interfere directly with potential binding sites on the C-terminal domain. In the Elo mutant, γE-crystallin is truncated, losing motif IV and thereby disrupting the whole of the C-terminal domain, although the N-terminal might be able to fold correctly. The fusion protein of the Elo mutant of γE-crystallin that lacks a folded C-terminal domain, does not interact with MIP. This could be due to loss of the proposed binding groove in the C-terminal domain, although it could also be due to decreased solubility of the truncated mutant protein.

Functional link between MIP and γE-crystallin: Both MIP and γ-crystallins are specifically expressed in the lens fibers. Five mutations resulting in genetic cataracts with a dominant phenotype have been identified in the murine γ-crystallin gene [34,36,39,55] and four in MIP [4-7]. Interestingly, both MIP and γE-crystallin associated cataracts have some similarities. When either MIP or γE-crystallin is mutated, the tight packing of lens fiber cells is disrupted and vacuolated cells are observed [4-7,36,39,55]. In addition, either in the absence of MIP [12] or in the presence of mutants of γE-crystallin [39,55] suture formation is perturbed in the mouse lens. There is also some correlation between γE-crystallin and MIP cataracts in time of appearance. In the Elo mouse, in which γE-crystallin is mutated, the cataract phenotype appears at embryonic day 12-13 [50,51,56], the same embryonic stage at which MIP expression begins in lens primary fibers [57] and at which the MIP Cat Fraser mouse mutant shows the cataract phenotype [58].

These observations suggest a possible functional link be-
between γE-crystallin and MIP in the maintenance of lens transparency. MIP has at least two potential roles in the lens. It can serve as a water channel, with consequences for lens hydration, and it can also act as a lens fiber adhesion protein [10,14,22]. There is evidence that the C-terminal peptide of MIP has a role in both these functions, affecting the constriction of the water channel and the ability of MIP subunits to form intercellular junctions [22,28]. Thus, an interaction between the MIP C-terminal peptide and either γE- or γF-crystallin could have major consequences for lens organization and physiology.

Evolutionary implications of γE-/γF-crystallin interaction with MIP and lens hydration: Although both γE- and γF-crystallin are expressed in mice and rats, the CRYGE and CRYGF genes in humans are non-functional pseudogenes [59-63]. Clearly, if there is a functional role for the MIP interaction with γE-/γF-crystallin in the rodent lens, it is absent from humans and must relate to a change in the human lens. In fact there are several ways in which the human lens differs from those of rodents. Most obvious is the “softness” or hydration of the human lens. Rodent lenses have high concentrations of γ-crystallins and may achieve protein concentrations of up to 60% wet weight. Their lenses are quite rigid and spherical with a short focal length and limited accommodative ability. In contrast human lenses have lower γ-crystallin content, lower overall protein concentration, and are much more flexible and accommodative. Furthermore, the organization of fiber cells and sutures is also significantly different in rodents and primates, with functional consequences for their focusing ability [64]. Thus, it is conceivable that the loss of γE- and γF-crystallin from the human lens reflects an evolutionary change in the regulation of organization and function of MIP, which in turn has major effects on the overall properties of the mammalian lens. Indeed, bird lenses lack the whole group of γA- to γF-crystallins and also have softer, accommodating lenses and a different organization of fiber cell packing from that seen in most mammals.

ACKNOWLEDGEMENTS

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