γE-crystallin Recruitment to the Plasma Membrane by Specific Interaction between Lens MIP/Aquaporin-0 and γE-crystallin

Jianguo Fan,1 Anna K. Donovan,1 Dolena R. Ledee,1 Peggy S. Zelenka,1 Robert N. Fariss,2 and Ana B. Chepelnisky1

PURPOSE. Major intrinsic protein (MIP), also called aquaporin-0, is essential for lens transparency and is specifically expressed in the lens fiber cell membranes. The goal of the current study was to identify and characterize proteins that interact with MIP and to elucidate the role of these interactions in MIP functions.

METHODS. The C-terminal 74-amino-acid fragment of MIP was used as bait to screen a rat lens cDNA yeast two-hybrid library. The full-length MIP was expressed as enhanced green fluorescent protein (EGFP)-tagged or myc-tagged proteins, respectively, in the RK13 rabbit kidney epithelial cell line. Protein-protein interactions were analyzed by communoprecipitation assays and visualized by confocal fluorescence microscopy.

RESULTS. γE-Crystallin, a water-soluble protein that is specifically expressed in lens fibers, was identified as a binding protein to the MIP C-terminal peptide. Communoprecipitation assays demonstrated that γE-crystallin interacts specifically with full-length MIP in mammalian cells. MIP did not interact with γD-crystallin, another member of the highly conserved γ-crystallin gene family. Confocal fluorescence microscopy demonstrated that MIP interacted with γE-crystallin in individual mammalian cells and that this interaction resulted in the recruitment of γE-crystallin from the cytoplasm to the plasma membrane.

CONCLUSIONS. These experiments provide the first demonstration of MIP interaction with other lens proteins at the molecular level and raise the possibility of a structural role of MIP in the organization of γ-crystallins in lens fibers. (Invest Ophtalmol Vis Sci. 2004;45:863–871) DOI:10.1167/iovs.03-0708

Lens major intrinsic protein (MIP), also known as aquaporin-0, is the most abundant protein of the ocular lens fiber membranes. It belongs to an ancient family of membrane channel proteins. 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. MIP may play a role in reducing the interfiber space, as tightly packed fibers are essential 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; lof), replacement of the last 61 amino acids (amino acids 203-263) at the MIP C-terminus by a transposon sequence (Cat Fr), deletion of MIP amino acids 121-175 (Hfr), and deletion of amino acids 46-49 (CatFdr) result in autosomal dominant cataracts and MIP trapping in the endoplasmic reticulum without being inserted into the plasma membrane.1–4 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.5,6 Water channel activity measurements of reconstituted lens membrane vesicles derived from Cat Fraser or MIP null mice, showed a marked decrease in water channel activity compared with those from the wild-type lens.7,8 However, the facts that some water channel activity remains, even in the absence of MIP, and severe changes in lens fiber structure with dominant phenotype are observed in MIP mutant mice suggest additional functions for MIP in the lens, such as maintenance of fiber structure and arrangement required for optimal focusing of the lens.8,9

MIP forms pH- and Ca2+-dependent water channels when expressed in Xenopus oocytes10 and pH- and voltage-dependent channels in mammalian and insect cells.11 MIP voltage-dependent channels in lipid bilayer vesicles are regulated by protein kinase-A–dependent phosphorylation.12 MIP may also function as an adhesion molecule and may play a role in gap junction formation.13–16 Both the N- and C-termini of MIP are located in the cytoplasmic side of the plasma membrane. The MIP C-terminal domain may play an important role in its physiological function. This domain is cleaved in cataractogenesis and aging,17–19 is serine phosphorylated,20 and may interact with calmodulin.21,22

To understand the role of the MIP C-terminus in MIP functions, we screened a lens yeast two-hybrid expression library with the MIP C-terminal domain as the bait to search for novel proteins expressed in the lens that may interact with this MIP domain. We identified γE-crystallin, a soluble protein that is also specifically expressed in the lens fibers, as a binding protein interacting with the MIP C-terminal domain. We provided biochemical evidence for the specific interaction between the full-length MIP and γE-crystallin in mammalian cells. We further demonstrated that this specific interaction results in the recruitment of γE-crystallin to the plasma membrane in mammalian cells expressing MIP. γ-crystallins are lens-fiber–specific soluble proteins that also play a critical role in maintaining lens transparency.23 Various mutations in different members of the γ-crystallin family have been linked to genetic cataracts in both mice and humans.24–32 However, the mechanisms by which γ-crystallins are involved in cataract formation are largely unknown. Thus, this study may provide a functional link between two distinct classes of lens proteins that are involved in genetic cataract formation.
MATERIALS AND METHODS

Chemicals and Reagents

TWEEN-20, Triton X-100, protease inhibitor cocktail for mammalian cells, and XGal were obtained from Sigma-Aldrich (St. Louis, MO). Media and sera for cell culture were purchased from Invitrogen (Carlsbad, CA). Agarose and DNA polymerase (Protein A/Protein G Plus Agarose and turbo Pfu polymerase) were obtained from Stratagene (La Jolla, CA). A polymerase chain reaction (PCR) purification kit and plasmid DNA purification were purchased from Qiagen (Valencia, CA). A sandwich membrane kit (Polyvinylidine Difluoride [PVDF] Membrane Sandwich Assembly Kit) was obtained from Invitrogen. A Western blot detection kit (Enhanced Chemiluminescence [ECL] Plus) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Non-fat dry milk and pre-stained molecular weight standards (Kaleidoscope) were obtained from Bio-Rad (Heracles, CA). All other chemicals were reagent grade and from standard commercial sources.

Antibodies

Antibodies against FLAG (M2) and anti-FLAG (M2) agarose conjugates were purchased from Sigma-Aldrich. A rabbit polyclonal anti-myc tag antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA). C-myc monoclonal antibody-agarose beads were obtained from Clontech (Palo Alto, CA). The anti-MIP rabbit polyclonal antibody MIP31 was prepared and affinity purified using a synthetic peptide derived from the mouse MIP amino acids 1-31. Rabbit polyclonal antibody against γ-crystallins was generously provided by Samuel Zigerl (the Laboratory of Mechanisms of Ocular Diseases, National Eye Institute).

Cell Lines

The RK13 (rabbit kidney) cell line was obtained from American Type Culture Collection (Manassas, VA) 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. Plasmid DNAs were propagated in Escherichia coli strain DH5α or DH10B and purified by ion exchange chromatography using plasmid kits (Plasmid Midi Endonuclease-free Maxi Kits) from Qiagen.

DNA Sequencing

Sequencing of plasmid DNA was performed with a commercial system (CEQ 2000XL; Beckman-Coulter), according to the manufacturer’s instructions.

Construction of MIP/Bait Plasmids

The MIP C-terminal bait plasmid pBDGal4-MIP-C74, which contains a MIP cDNA fragment encoding the C-terminal 74 amino acids (amino acids 190-263), was constructed as follows. The MIP C-terminal cDNA fragment was amplified from a full-length mouse MIP cDNA plasmid by polymerase chain reaction (PCR) using turbo Pfu DNA polymerase (Stratagene) with the amplimers 5'-ATCAGATGGTGTCGTCATGCCGCGCCCCCGAAC-3' (forward) and 5'-ATGCTCGACCCCGGGCCTGTCCGAC-5' (reverse). The PCR product was digested with EcoRI and SalI restriction enzymes, purified with a PCR purification kit (Qiagen), and subsequently cloned into the parental bait plasmid pBDGal4-hcam (Stratagene) at EcoRI and SalI sites. The mouse MIP full-length bait plasmid pBDGal4-MIP-F was constructed in the same manner, except that the amplimers used for PCR amplification of the full-length MIP cDNA were 5'-ATCAGATGGTGTCGACTACGGGCGCGCCCCCGAAC-3' (forward) and 5'-ATGCTCGACCCCGGGCCTGTCCGAC-5' (reverse). DNA sequences of the insert and the junction regions flanking the insert in these plasmids were verified by DNA sequencing.

Construction of MIP/EGFP, MIP/Myc, FLAG/γ-crystallin, or HcRed/γ-crystallin Fusion Vectors

To construct an enhanced green fluorescent protein (EGFP) fusion plasmid of MIP, the full-length MIP cDNA fragment was cut from the plasmid pBDGal4-MIP-F with EcoRI and SalI restriction enzymes and purified on 1.2% agarose gel. This fragment was subsequently cloned into either pEGFP-C2 or pEGFP-N2 (Clontech) at EcoRI and SalI sites, producing plasmid pEGFP-MIP (i.e., EGFP is fused to the N-terminal site of MIP) or pMIP-EGFP (i.e., EGFP is fused to the C-terminal site of MIP), respectively.

To construct the myc/MIP fusion vector pmyc-MIP, the entire MIP coding sequence was amplified from the plasmid pBDGal4-MIP-F with PCR and turbo Pfu DNA polymerase, using the following amplifiers: 5'-ATATGGATCCGGTTCTGGATCAATGTGGGAACTTCGGTCTGC-3' (forward) and 5'-ATCAGAATTCTTACAGGGCCTGAGTC TTCAGTTC-3' (reverse). The PCR product, after digestion with BamHI and EcoRI restriction enzymes, was cloned into the BamHI and EcoRI sites of pCMVTag3B (Stratagene), producing the vector pmyc-MIP (i.e., myc tag at the N-terminal site of MIP). To construct the myc/MIP fusion vector pMIPmyc (i.e., myc tag at the C-terminal site of MIP), the full-length mouse MIP cDNA fragment, cut from the plasmid pBDGal4-MIP-F with restriction enzymes EcoRI and SalI, was purified on 1.2% agarose gel, cloned into the EcoRI and SalI sites of the plasmid pCMVTag5C (Stratagene).

To construct the FLAG/γ-crystallin fusion expression plasmid pFLAG-γ-cr (i.e., FLAG tagged at the N-terminus of γ-crystallin), the full-length rat γ-crystallin cDNA fragment (0.5 kb), cut from pADc12-1b (a target cDNA clone obtained from the yeast two-hybrid screening of rat lens cDNA library), was cloned into EcoRI and Xhol restriction enzymes, purified on 1.2% agarose gel and cloned into the same restriction sites in the plasmid pCMV-Tag2B (Stratagene). The FLAG/γ-crystallin fusion expression plasmid pFLAG-γ-cr was constructed as follows. The full-length rat γ-crystallin cDNA fragment (0.5 kb) was isolated from pAD2B2B (a target cDNA clone obtained from the yeast two-hybrid screening of rat lens cDNA library) by digestion with EcoRI and Xhol restriction enzymes. After purification by agarose gel electrophoresis, the fragment was cloned into the plasmid pCMV-Tag2B at the EcoRI and Xhol sites, producing pFLAG-γ-cr.

To construct the HcRed/γ-crystallin fusion plasmid pHcRed-γ-cr, the full-length rat γ-crystallin cDNA was amplified, using PCR with turbo Pfu DNA polymerase, from pADc12-1b with the following amplifiers: (forward) 5'-ATCATGATGGTGTCGTCATGCCGCGCCCCCGAAC-3' and (reverse) 5'-TATTAGTTCGACCACTA- GAAATCCATGATTCTCCT CAG-3'. After digestion with BglII 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-crystallin fusion plasmid pHcRed-γ-cr, the full-length γ-crystallin cDNA fragment (0.5 kb) was cut from pAD2B2B pADc12-1b with restriction enzymes BglII and SalI and purified on 1.2% agarose gel, cloned into the BglII and SalI restriction sites of pHcRed1-C1 (Clontech).

All expression vectors constructed are under the 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.

Construction and Preparation of the Rat Lens cDNA Library for Yeast Two-Hybrid Screening

mRNAs were isolated and purified from 18-day rat embryo lenses and reverse transcribed into cDNAs with an oligo dT primer. After addition of adapter sequences with appropriate restriction sites, the double-stranded cDNAs were size fractionated and ligated into the HybriZAP
2.1 vector (Stratagene). The ligation products were packaged by using a packaging extract (Gigapack III Gold; Stratagene). The resultant λ-phage library was subsequently amplified, excised into pADGal4-H9261 phagemids, and amplified again. The library plasmid DNA (pADGal4-H9261) was transferred into E. coli L (bed volume) of anti-FLAG M2 antibody-agarose beads (cat. no. 18840; Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (4\(\text{Cl}^{-}\),6\(\text{Cl}^{-}\)-diamidino-2-phenylindole dihydrochloride (DAPI; pH 7.3), for 30 minutes at room temperature. Cells were washed three times with PBS and counterstained with 2 mL per dish of 0.5% bovine serum albumin, 0.2% Tween-20, 0.05% sodium azide, and 1 \(\mu\)g/mL 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; pH 7.3), for 10 minutes at room temperature. Cells were washed three times with PBS and mounted (Gel Mount; BioMeda Corp., Foster City, CA), an aqueous mounting medium containing a proprietary anti-photograph bleaching reagent. The cells were imaged using a confocal microscope (model TCS SP2) with a 40× 0.85-NA HCX Plan Apo CS objective lens (all from Leica, Deerfield, IL). EGFP fluorescence was imaged at the wavelengths of 458, 476, and 488 nm (excitation) and 510 to 540 nm (emission). HcRed fluorescence was imaged at the wavelengths of 568 nm (excitation) and 600 to 650 nm (emission). DAPI fluorescence was imaged at the wavelengths of 351 and 364 nm (excitation) and 400 to 500 nm (emission). To minimize crosstalk between channels, fluorochromes were excited sequentially rather than simultaneously, using the sequential scan mode of the confocal microscope.

**Results**

**Identification of \(\gamma\)-E-crystallin as a Binding Protein to the MIP C-Terminal Domain**

A CDNA fragment encoding 74 amino acids of the mouse MIP C-terminal domain (amino acids 190-263) was cloned into the parental bait plasmid (pBDGal4Cam) of the yeast two-hybrid system and subsequently used as bait to screen a yeast two-hybrid library containing cDNAs isolated from 18-day rat embryo lenses (see the Materials and Methods section for details). The bait plasmid (pBDGal4-MIP-C74) and the library DNA were introduced in a sequential manner into yeast strain YRG-2. After growth selection of cells in medium lacking tryptophan, leucine, and histidine, positive clones were identified by a filter-lift assay that measures \(\beta\)-galactosidase activity (blue). Identity of the target CDNA present in the plasmid from these positive clones was obtained by DNA sequencing, followed by a BLAST search of DNA/protein databases. Two of the positive, in-frame clones obtained with the MIP C-terminal fragment as

**Western Blot Analysis**

After electrophoresis, the proteins were electro-transferred to PVDF membranes and the latter were probed with either anti-myc or anti-MIP or anti-\(\gamma\)-crystallin antibody followed by a second antibody conjugated with peroxidase or with anti-FLAG antibody conjugated with peroxidase (no secondary antibody step). Specific proteins were then visualized by treatment of the membranes with the enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech) followed by exposure to x-ray films.
bait encode γ-crystallin (containing full-length cDNA). No other γ-crystallin (i.e., γA, γB, γC, γD, or γE) was identified with this MIP C-terminal bait, although all these γ-crystallins are expressed in the lens yeast two-hybrid library, as determined by cloning and sequencing. Interaction between MIP C-terminal and γE-crystallin in yeast was confirmed by the yeast two-hybrid assay, in which bait and target plasmids were co-transformed into yeast followed by verification of β-galactosidase activity (blue) of transformed yeast cells. The results indicate that γE-crystallin may interact with MIP and that the interaction may involve the C-terminus of MIP.

**Specific Interaction between Full-Length MIP and γE-crystallin in Mammalian Cells**

To demonstrate the specific interaction between MIP and γE-crystallin in mammalian cells, mammalian expression vectors for expressing the full-length MIP as myc- and EGFP-tagged proteins and the full-length γE- or γD-crystallin as FLAG- and HcRed-tagged proteins were constructed as indicated in Materials and Methods (Fig. 1). The EGFP-tag was placed at either the C- or N-terminal end of MIP, generating the fusion proteins MIP-EGFP and EGFP-MIP, respectively (Figs. 1A, 1B). Similarly, the myc tag was placed at either the N- or C-terminal end of MIP, generating the fusion proteins myc-MIP and MIP-myc, respectively (Figs. 1C, 1D). The FLAG tag was placed at the N-terminal end of γE- or γD-crystallin, generating the fusion proteins FLAG-γE-crystallin and FLAG-γD-crystallin, respectively (Figs. 1E, 1F). The HcRed tag was fused to the N-terminal side of γE- or γD-crystallin, producing the fusion proteins HcRed-γE-crystallin and HcRed-γD-crystallin, respectively (Figs. 1G, 1H).

Coimmunoprecipitation assays were performed to provide the biochemical evidence for the specific interaction between full-length MIP and γE-crystallin in mammalian cells. To this end, myc-tagged MIP (either myc-MIP or MIP-myc) and FLAG-tagged γE-crystallin expression vectors were cotransfected into rabbit kidney epithelial (RK13) cells (Fig. 2, lanes 1 and 2) along with various controls (lanes 3-5 and 7). Total cellular extracts from the transfected cells were immunoprecipitated with either anti-myc (Figs. 2A, 2B) or anti-FLAG (Figs. 2C, 2D) antibody-agarose conjugate. The immunoprecipitated proteins were resolved on a denaturing SDS-polyacrylamide gel. After transfer of proteins to a PVDF membrane, the immunoprecipitated proteins were visualized by Western blot analysis with either anti-myc or anti-FLAG antibody.

Coimmunoprecipitation using anti-myc antibody was examined first. The efficacy of IP of myc-tagged proteins with anti-myc antibody agarose conjugate was tested with IP followed by Western blot with anti-myc antibody (Fig. 2A). The protein...
levels of the immunoprecipitated myc-tagged MIP from cotransfected cells (Fig. 2A, lanes 1 and 2) were comparable to each other and to those from control cells into which a single expression vector was transfected (Fig. 2A, lanes 4 and 5). Myc-tagged MIP protein was not detected in cells that were transfected with FLAG-tagged γE-crystallin alone (Fig. 2A, lane 3) or in cells that were mock transfected (Fig. 2A, lane 7). The relative levels of the immunoprecipitated myc-tagged proteins (Fig. 2A, lanes 1–2, 4, 5) were comparable to those detected in total cellular extracts (Fig. 2E, lanes 1, 2, 4, 5). These results indicate that myc-tagged proteins were expressed at comparable levels and were immunoprecipitated with similar efficacy in cotransfected cells as well as in control cells. When the anti-myc immunoprecipitates were analyzed by Western blot using anti-FLAG antibody (Fig. 2B), the FLAG-tagged γE-crystallin was coimmunoprecipitated with either myc-MIP (Fig. 2B, lane 1) or MIP-myc (Fig. 2B, lane 2). The specificity of the coimmunoprecipitation reaction was demonstrated by the absence of coimmunoprecipitation in control experiments in which cells were transfected with only a single expression vector (Fig. 2B, lanes 3–5) or when cells were mock transfected (Fig. 2B, lane 7).

Next, the reciprocal coimmunoprecipitation using the anti-FLAG antibody was performed in a similar manner. The efficacy of IP of FLAG-tagged proteins with anti-FLAG antibody agarose conjugate was tested with IP followed by western blotting with anti-FLAG antibody (Fig. 2C). The protein levels of the immunoprecipitated FLAG-tagged γE-crystallin from cotransfected cells (Fig. 2C, lanes 1, 2) were similar to each other and to those from control cells into which a single expression vector was transfected (Fig. 2C, lane 3). FLAG-tagged γE-crystallin protein was not detected in cells that were transfected with myc-tagged MIP vectors alone (Fig. 2C, lanes 4, 5) or in cells that were mock transfected (Fig. 2C, lane 7). The relative levels of the immunoprecipitated FLAG-tagged proteins (Fig. 2C, lanes 1–3) were very similar to those detected in total cellular extracts (Fig. 2F, lanes 1–3). These results indicate that FLAG-tagged γE-crystallin was expressed at comparable levels and was immunoprecipitated with similar efficiency in cotransfected cells as well as in control cells. When the anti-FLAG immunoprecipitates were analyzed by Western blot with anti-myc antibody (Fig. 2D), the myc-tagged MIP protein was coimmunoprecipitated with FLAG-tagged γE-crystallin (Fig. 2D, lanes 1, 2). The specificity of the coimmunoprecipitation reaction was demonstrated by the absence of coimmunoprecipitation in control experiments in which cells were transfected with only a single expression vector (Fig. 2D, lanes 3–5) or when cells were mock transfected (Fig. 2C, lane 7). The appearance of a double band for MIP-myc (Figs. 2A, 2D, 2E, lane 2) is probably due to the use of the commercially available expression vectors (Stratagene), which tend to express different C-terminally tagged proteins as a double band (Fan J, et al., unpublished data, 2003).

Thus, our data showed that MIP is coimmunoprecipitated with γE-crystallin through an anti-FLAG antibody and, vice versa, that γE-crystallin is coimmunoprecipitated with MIP through an anti-myc antibody. These results indicate that full-length MIP specifically interacts with γE-crystallin in mammalian cells.

Involvement of the MIP C-Terminal Domain in Interaction between MIP and γE-crystallin

Interaction between MIP and γE-crystallin was further studied in coimmunoprecipitation experiments using EGFP-tagged MIP and FLAG-tagged γE-crystallin expression vectors. EGFP-tagged MIP (either EGFP-MIP or MIP-EGFP, Fig. 1) and FLAG-tagged γE-crystallin vectors were cotransfected into RK13 cells. Total cellular extracts from the transfected cells were prepared and immunoprecipitated with anti-FLAG antibody-agarose conjugate. The immunoprecipitated proteins were resolved on a denaturing SDS-polyacrylamide gel. After transfer of proteins to a PVDF membrane, the immunoprecipitated proteins were visualized by Western blot with various antibodies. The efficacy of IP of FLAG-tagged proteins with anti-FLAG agarose conjugate was verified with IP followed by Western blot with both an anti-FLAG antibody (Fig. 3A). The protein level of the immunoprecipitated FLAG-tagged γE-crystallin was comparable in various transfection experiments (Fig. 3A, lanes 1–3), indicating that FLAG-tagged proteins were expressed at comparable levels and were immunoprecipitated with similar efficacy among different samples. Western blot of the anti-FLAG immunoprecipitates with an anti-γ-crystallin antibody (Fig. 3C) confirmed this result and, in addition, verified the identity of the FLAG-tagged γE-crystallin fusion protein. The expression levels of EGFP-tagged MIP (EGFP-MIP or MIP-EGFP) in cells transfected with these plasmids were also comparable, as verified by confocal fluorescence microscopy (Figs. 4B, 4G). Under these conditions, when the anti-FLAG immunoprecipitates were analyzed by Western blot with the anti-MIP antibody MIP31 (Fig. 3B), an EGFP-MIP fusion protein (~50 kDa) was detected in the immunoprecipitated samples prepared from cells cotransfected with EGFP-MIP and FLAG-γE-crystallin expression vectors (Fig. 3B, lane 1). In contrast, no MIP fusion protein band was detected in immunoprecipitates from cells cotransfected with MIP-EGFP and FLAG-γE-crystallin expression vectors (Fig. 3B, lane 2). Negative results were also obtained when cells were cotransfected with the parental EGFP plasmid (i.e., no MIP fusion) and FLAG-γE-crystallin expression vectors (Fig. 3B, lane 3), further confirming the specificity of the coimmunoprecipitation reaction. These results indicate that EGFP-MIP but not MIP-EGFP interacts with FLAG-tagged γE-crystallin, suggesting
that a bulky EGFP moiety at the C-terminus of MIP may interfere with the interaction between MIP and γE-crystallin. These data, along with the fact that the C-terminus of MIP was actually used as the bait molecule for the yeast two-hybrid screening for MIP-binding proteins, strongly support the notion that C-terminus of MIP may play a critical role in the interaction between MIP and γE-crystallin.

Lack of Interaction between MIP and γD-crystallin

Because γE-crystallin is a member of the highly conserved γ-crystallin gene family, we chose γD-crystallin to test whether MIP interacts with other members of the γ-crystallin family. Possible interaction between MIP and γD-crystallin was investigated in a manner similar to that described earlier for interaction between MIP and γE-crystallin (Fig. 2). RK13 cells were cotransfected with myc-MIP and FLAG-γD-crystallin, and cell extracts from cotransfected cells were immunoprecipitated with either anti-myc or anti-FLAG antibody, followed by Western blot with anti-myc or anti-FLAG antibodies. In the coimmunoprecipitation experiment using anti-myc antibody, FLAG-tagged γD-crystallin did not coimmunoprecipitate with myc-MIP (Fig. 2B, lane 6), whereas FLAG-tagged γE-crystallin coimmunoprecipitated with myc-MIP (Fig. 2B, lane 1), although the expression levels of FLAG-tagged γE-crystallin and γD-crystallin (Fig. 2F, compare lanes 6 and 1) and efficiency of IP for myc-MIP (Fig. 2A, compare lanes 6 and 1) were very similar. In the reciprocal coimmunoprecipitation experiment using anti-FLAG antibody, myc-MIP did not coimmunoprecipitate with FLAG-tagged γD-crystallin (Fig. 2D, lane 6), whereas myc-MIP coimmunoprecipitated with FLAG-tagged γE-crystallin (Fig. 2D, lane 1). Again, the expression levels of myc-MIP (Fig. 2E, compare lanes 6 and 1) and efficacy of IP for FLAG-tagged γE-crystallin and γD-crystallin (Fig. 2C, compare lanes 6 and 1) were very similar.

Furthermore, in a separate coimmunoprecipitation experiment involving EGFP-tagged MIP and either FLAG-tagged γE-crystallin or FLAG-tagged γD-crystallin, the protein level of the immunoprecipitates and IP efficacy of FLAG-tagged γD-crystallin were comparable to those of FLAG-tagged γE-crystallin (Fig. 2E, compare lanes 4, 5 with lanes 1–3). Expression levels of EGFP-MIP or MIP-EGFP in cells transfected with these vectors were also comparable, as determined by confocal fluorescence microscopy (Figs. 4B, 4G, described later). Under these conditions, FLAG-tagged γD-crystallin did not coimmunoprecipitate with either EGFP-MIP or MIP-EGFP (Fig. 3B, lanes 1, 2, 3), whereas FLAG-tagged γE-crystallin coimmunoprecipitated with EGFP-MIP (Fig. 3B, lane 1). These results further confirmed that MIP interacts with γE-crystallin but does not interact with γD-crystallin, although they are highly homologous to each other. Indeed, the lack of interaction between MIP and γD-crystallin serves as a good physiological control for the specificity of the interaction between MIP and γE-crystallin.

Recruitment of γE-crystallin to the Plasma Membrane by MIP

To determine the cellular localization of interaction between MIP and γE-crystallin in mammalian cells, MIP and γE-crystallin were expressed as EGFP- and red fluorescent protein (HcRed)-tagged proteins, respectively, and the interaction between MIP and γE-crystallin was visualized in individual mammalian cells by confocal fluorescence microscopy.

RK13 cells were transiently cotransfected with the EGFP/MIP fusion vector (either pEGFP-MIP or pMIP-EGFP) and the expression vector pHcRed-γE-cry in which HcRed was fused at the N-terminal end of γE-crystallin. The transfected cells were examined by confocal fluorescence microscopy. When RK13 cells were cotransfected with pEGFP-MIP and pHcRed-γE-cry (Figs. 4A–E), the EGFP fluorescence was concentrated to the plasma membrane (Fig. 4B), although cytoplasmic and nuclear fluorescence was quantified separately and plotted as a function of distance along the path. Blue dashed lines (E, J, T): approximate location of the plasma membrane. Photographs and spatial quantification graphs shown are representative (>90%) of those obtained from at least 12 different fields in at least three independent experiments. Bar, 10 µm.
expression was also evident. The HcRed fluorescence was also localized to the plasma membrane (Fig. 4C) although low HcRed fluorescence in the cytoplasm and strong HcRed fluorescence in the nucleus were also observed. When the EGFP and HcRed fluorescence images of the same field were superimposed (Fig. 4D), a yellow signal around the plasma membrane was observed, indicating that MIP and γE-crystallin co-localized to the plasma membrane. The colocalization of MIP and γE-crystallin was further demonstrated by spatial quantification of both EGFP and HcRed fluorescence along a path that goes across the plasma membrane (Fig. 4E). The EGFP and HcRed fluorescence showed almost identical profiles along the path, both of which peaked at the same location (most likely the plasma membrane).

In contrast, cells cotransfected with MIP-EGFP and pHcRed-γE-cry showed different results (Figs. 4F–J). EGFP fluorescence was localized to the plasma membrane, similar to cells cotransfected with EGFP-MIP and pHcRed-γE-cry (Fig. 4A). Cyttoplasmic and nuclear EGFP fluorescence was significantly less in MIP-EGFP-transfected cells than in EGFP-MIP-transfected cells. However, the HcRed fluorescence was completely diffused throughout the cells (Fig. 4H). No plasma membrane localization of γE-crystallin was evident. The absence of yellow-membrane-associated fluorescence in these cells (Fig. 4I) indicates that γE-cry did not colocalize with MIP in cells cotransfected with MIP-EGFP and pHcRed-γE-cry. This result was confirmed by a spatial profile measurement (Fig. 4J) that showed that EGFP fluorescence did not overlap with HcRed fluorescence at the vicinity of the plasma membrane. These data indicate that the presence of a relatively bulky EGFP moiety at the C-terminus of MIP interfered with the interaction between MIP and γE-crystallin. This result was confirmed by a separate coimmunoprecipitation experiment that showed that EGFP-MIP, but not MIP-EGFP, coimmunoprecipitated with FLAG-tagged γE-crystallin in cotransfected RK13 cells (Fig. 3B).

In contrast, the absence of the relatively small myc tag at the C-terminus of MIP did not affect the interaction between MIP and γE-crystallin (Figs. 2B, 2D, lanes 1 and 2). These data, together with the fact that the C-terminal peptide of MIP was actually used as the bait for yeast two-hybrid screening to identify MIP interacting proteins, suggest that the C-terminus of MIP may play a key role in its interaction with γE-crystallin.

In a control experiment, RK13 cells were cotransfected with pEGFP and pHcRed-γE-cry (Figs. 4K–O). In the absence of the MIP moiety, EGFP showed a diffuse intracellular distribution typical of a soluble cytoplasmic protein (Fig. 4L). HcRed fluorescence also displayed diffuse distribution under these conditions (Fig. 4M). The superimposed image of EGFP and HcRed fluorescence (Fig. 4N) as well as the spatial profile measurement (Fig. 4O) showed that there is no localization of either EGFP or HcRed on the plasma membrane.

**Lack of Recruitment of γD-crystallin to the Plasma Membrane by MIP**

As shown earlier (Figs. 2, 3), MIP does not interact with γD-crystallin. This lack of interaction between MIP and γD-crystallin was further demonstrated using confocal fluorescence microscopy. RK13 cells were cotransfected with EGFP-MIP and HcRed-tagged γD-crystallin or γE-crystallin and then visualized by confocal fluorescence microscopy. As shown in Figure 4, EGFP-MIP colocalized with γE-crystallin and recruited γE-crystallin to the plasma membrane (Figs. 4A–E). In contrast, in cells coexpressing EGFP-MIP and HcRed-tagged γD-crystallin, only EGFP-MIP localized to the plasma membrane (Figs. 4Q, 4S), whereas HcRed-tagged γD-crystallin displayed a diffuse intracellular localization pattern without any membrane localization (Figs. 4R, 4S). This conclusion was confirmed by spatial quantification shown in Figure 4T. These data indicate that MIP does not interact with γD-crystallin in RK13 cells, and is thereby unable to recruit γD-crystallin to the plasma membrane.

In summary, the data presented in this article demonstrate that MIP interacts specifically with γE-crystallin in mammalian cells and this interaction results in the recruitment of γE-crystallin from the cytoplasm to the plasma membrane.

**DISCUSSION**

**MIP C-Terminus as Bait for Yeast Two-Hybrid Screening**

In this study, we used a rat lens cDNA yeast two-hybrid library to screen for lens proteins that interact with MIP. The high hydrophobicity of MIP, a membrane channel protein, may interfere with its transport to the nucleus, required for the yeast two-hybrid interaction. Therefore, the full-length MIP may not be a suitable bait for yeast two-hybrid screening. The relatively hydrophilic C-terminus of MIP is located in the cytoplasmic side of the plasma membrane and is most likely involved in the MIP interaction with other proteins. Therefore, the 74-amino-acid C-terminus of MIP, rather than the full-length MIP molecule, was used as the bait for the yeast two-hybrid screening. We identified the lens fiber-specific γE-crystallin as a binding protein to the lens MIP/aquaporin-0 C-terminal domain.

Although the full-length MIP bait construct was not used for the yeast two-hybrid screening, it was tested in the yeast two-hybrid assay. In most cases, it gave significantly less qualitative interaction signal (lighter-blue-stained colonies or longer times required for appearance of blue in the colonies) than when the C-terminal MIP fragment was used as the bait. This confirms our hypothesis that the full-length MIP may not be a good bait molecule for screening the yeast two-hybrid library. The interaction between γE-crystallin and the full-length MIP molecule in mammalian cells was subsequently confirmed by both coimmunoprecipitation assays and confocal fluorescence microscopy.

**MIP Integration in the Plasma Membrane with a Long Hydrophilic Tag at the MIP N- or C-Terminus**

To visualize the cellular localization of MIP in mammalian cells, we inserted an EGFP (265 amino acids) tag either at the N- or C-terminus of MIP. The addition of this long hydrophilic tail—almost the same length as MIP—at either end of the MIP molecule, still allows the hybrid protein to be inserted in the plasma membrane of epithelial cells. Even though the blocking of the MIP N-terminal does not interfere with plasma membrane insertion, we also observed some dense structures in the cytoplasm, suggesting that a free N-terminal is necessary for an efficient insertion in the plasma membrane. It is interesting that the six mutations reported in the MIP protein linked to genetic cata-�actas and that prevent MIP integration in the plasma membrane, localize to hydrophobic transmembrane domains.1 6 These results suggest that the MIP transmembrane domain amino acid sequence is critical for the ability of MIP to be inserted into the plasma membrane and that addition of hydrophilic amino acids at the N- or C-terminal cytoplasmic domains does not interfere with MIP insertion in the plasma membrane.

**Involvement of MIP C-Terminus in the Interaction between MIP and γE-Crystallin**

The coimmunoprecipitation experiments showed that both myc-MIP and MIP-myc (i.e., MIP with a short myc-tag, a 10-
amino-acid peptide, at either the N- or C-terminal end of MIP) interacted with γE-crystallin (Fig. 2). However, EGFP-MIP (with the bulky EGFP moiety at the N-terminal side of MIP), but not MIP-EGFP (with the bulky EGFP moiety at the C-terminal side of MIP), interacted with γE-crystallin (Fig. 3). These results were confirmed further by confocal fluorescence microscopy, which showed that EGFP-MIP, but not MIP-EGFP, interacted with γE-crystallin, although both EGFP-MIP and MIP-EGFP were able to insert into the plasma membrane when transfected into rabbit kidney epithelial cells. These data suggest that MIP integration in the plasma membrane is not sufficient for interaction with γE-crystallin; a bulky EGFP tag at the C-terminus of MIP interferes with this interaction. These results suggest that the C-terminus of MIP may play a key role in its interaction with γE-crystallin. In contrast, γE-crystallin is able to interact with MIP when a FLAG tag is attached to either the N- or C-terminus of γE-crystallin (Fan et al., unpublished data, 2003), suggesting the addition of the short FLAG tag (a nine-amino-acid peptide) may not interfere with the tridimensional structure of γE-crystallin necessary for the interaction with MIP.

Interaction of MIP with γE-crystallin but Not with γD-crystallin

Both the biochemical (Figs. 2, 3) and confocal fluorescence microscopy (Fig. 4) data presented in this article show that myc-tagged MIP (either myc-MIP or MIP-myc) and EGFP-MIP transfected into rabbit kidney epithelial cells are able to interact with γE-crystallin but not with γD-crystallin. γE-crystallin is a member of the highly conserved family of γ-crystallins,25-35 specifically expressed in the lens fibers and differentially regulated during lens development.36-38 The particular function of each member of the crystallin family in the lens is not known. γE- and γD-crystallins are 91% identical at the amino acid level and the nonconserved amino acids are mainly in the third Greek key motif.25-35 The 14 different amino acids in γE- and γD-crystallins result in differences in the intramolecular ion pairs on the surfaces of both γ-crystallins.57 The clustering of four histidines between positions 111 and 125 in the rat γE-crystallin but the presence of only one histidine in the same region of rat γD-crystallin may result in differences in their intermolecular interactions.57 Therefore, different amino acids in γE- and γD-crystallins may be available for interaction with other proteins, with only γE-crystallin being able to interact with MIP under the experimental conditions we have used. In addition, our data are consistent with the view that closely related γ-crystallin family members may have distinct functions.

Recruitment of γE-crystallin to the Plasma Membrane by MIP

The addition of an HeRed tail to γE-crystallin allowed us to visualize the recruitment of γE-crystallin to the plasma membrane by MIP. Our results show that integration of MIP into the plasma membrane and an unblocked MIP C-terminus is necessary for recruitment of γE-crystallin to the plasma membrane. A calcium-ion–binding site at the γ-crystallin Greek key motif has been identified.36 As MIP water channel is calcium regulated, it is tempting to speculate that γE-crystallin calcium binding has a role in regulating MIP channel activity.

Functional Link between MIP and γ-crystallins

Both MIP and γ-crystallins are specifically expressed in the lens fibers. γE-crystallin plays a role in transparency of the mouse lens. Five mutations resulting in genetic cataracts with a dominant phenotype have been identified in the murine γE-crystallin gene.24-26,29,39 The tightly packed lens fiber structure is disrupted and appearance of vacuolated cells is observed when either MIP or γE-crystallin is mutated.1-4,26,29,39 It is also interesting to point out that perturbation of suture formation is observed when either MIP is not expressed50 or the γE-crystallin is mutated.50-52 Furthermore, γ-crystallins are selectively lost in the Cat Fraser MIP mutant.43 The Elo cataract is a mouse genetic cataract that results from a mutation in the γE-crystallin gene, in which the cataract phenotype appears at embryonic days 12 to 13.41-43 It is noteworthy that this is the same embryonic stage at which MIP begins to be expressed in the lens primary fibers44 and is the same stage at which the MIP Cat Fraser mouse mutant shows the cataract phenotype.45 Indeed, we have found that MIP does not interact with the γE-crystallin Elo mutant, whereas it interacts with its wild-type counterpart (Fan et al., unpublished data, 2003). Our results demonstrate for the first time specific interaction between MIP and γE-crystallin, providing evidence of a functional link between MIP and γ-crystallins. Our data also suggest that interaction between MIP and γE-crystallin may have important implications for how MIP and γ-crystallins are involved in lens cataractogenesis.

It is intriguing that γE-crystallin is a pseudogene in humans, resulting in lack of expression of this gene.54-56,59-61 Our results suggest that subtle amino acid changes may affect the interaction of γ-crystallins with MIP. Therefore, evolutionary changes in the amino acid sequences of γ-crystallins and/or MIP expressed in humans may result in the interaction of human MIP with another member of the γ-crystallin family instead of γE-crystallin. Future research will help elucidate this paradigm.

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References


