Interaction of major intrinsic protein (aquaporin-0) with fiber connexins in lens development

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Accepted 16 October 2003
Journal of Cell Science 117, 871-880 Published by The Company of Biologists 2004
doi:10.1242/jcs.00945

Summary

We observed that chick lens-fiber gap-junction-forming proteins, connexin (Cx) 45.6 and Cx56, were associated with an unknown protein, which was then identified as major intrinsic protein (MIP), also known as aquaporin-0 (AQP0), the most abundant membrane protein in lens fibers. A 1063 bp cDNA of chick MIP(AQP0) was identified that encodes a 262 amino acid protein with a predicted molecular weight of 28.1 kDa. Dual immunofluorescence and confocal microscopy of sagittal and coronal sections of the lens tissues showed that MIP(AQP0) consistently localized with gap junction plaques formed by Cx45.6 and Cx56 during the early stages of embryonic chick lens development. Immunoprecipitation combined with immunoblotting analyses revealed that MIP(AQP0) was associated with Cx45.6 and Cx56 at these developmental stages. The specificity of this interaction was further confirmed with the silver staining of the protein components of immunoprecipitates. The pull-down analysis of lens lysates revealed that C-terminus of MIP(AQP0) probably interacted with these two fiber connexins. In late embryonic and adult lenses, however, uniform co-distribution of MIP(AQP0) and fiber connexins was largely disrupted, except for the area surrounding the actively differentiating bow regions, as was revealed by immunofluorescence and immunoprecipitation experiments. The interaction of MIP(AQP0) with lens fiber connexins in differentiating lens cells but not in mature lens fibers suggests a potential role for MIP(AQP0) in the facilitation of fiber connexins for the formation of gap junctions during lens development.

Key words: Gap junction, Connexin, MIP(AQP0), Chick lens, Lens development

Introduction

The vertebrate lens is composed of two ectodermally derived cell types: lens epithelial cells and fiber cells, which differentiate from lens epithelial cells to form the bulk of the lens. The lens epithelial cells experience a complex program of genetic and morphological changes as part of their differentiation, which continues throughout the life of the organism as the lens continues its growth at the equator region. The daughter fiber cells elongate and are pushed towards the lens core, lose their nuclei and organelles, and synthesize tissue-specific proteins such as MIP(AQP0). Unlike other organs, the lens has no blood supply and has minimized oxidative phosphorylation in order to avoid heme-containing proteins and mitochondria that would disrupt the visual field. Thus, the fibers in the center of the lens are uniquely dependent for osmotic and metabolic homeostasis on a highly developed network of gap-junction-mediated intercellular communication to facilitate the exchange of ions and metabolites (Mathias et al., 1997; McAvoy et al., 1999).

Gap junctions are clusters of transmembrane channels permitting molecules with a molecular weight of <1 kDa to pass directly from the cytoplasm of one cell to adjacent cells. The chick lens is known to express three different connexins (Cx43, Cx45.6 and Cx56) that are structural components of gap junctions. Cx43 is a component of gap junctions joining lens epithelial cells, differentiating and elongating lens fibers at the lens equator (Musil et al., 1990). By contrast, Cx45.6 and Cx56 appear to be predominantly expressed in the lens fibers and colocalized in the same gap-junctional plaques (Rup et al., 1993; Jiang et al., 1994).

The most abundant membrane protein of lens fibers, MIP(AQP0), shares no sequence homology with connexins. Previous studies show that MIP(AQP0) expression seems to localize to thin, asymmetric membrane interactions, distinguishable from the thicker, symmetric junctions that contain connexins in the adult bovine lens (Gruijters et al., 1987). When analysed in the paired *Xenopus* oocytes (Kushmerick et al., 1995) and transfected BHK cells (Miller et al., 1992), MIP(AQP0) does not form gap-junction-like channels but is instead a member of the aquaporin protein family, with water-channel activity (Ehring et al., 1990; Fischbarg, 1995; Kushmerick et al., 1995). However, when expressed in oocytes lens, MIP(AQP0) only increases the osmotic water permeability twofold (Mulders et al., 1995; Kushmerick et al., 1995; Ishibashi and Sasaki, 1998), which is significantly lower than the 42-fold increase achieved by aquaporin-1 in erythrocytes (Chandy et al., 1997). Thus, other unidentified functions have been inferred for MIP(AQP0) in the lens (Kushmerick and Varadaraj, 1998). The initiation of MIP(AQP0) expression occurs soon after the embryonic lens is committed to the process of terminal differentiation to form elongating fibers, coincidentally with the assembly of lens fiber gap junctions (Yancey et al., 1988). In the adult ovine lens, MIP(AQP0) appears to associate transiently with lens fiber gap

junctions at the narrow zone of enlarged junctional plaques and newly formed ball-and-socket domains (Gruijters, 1989). SDS-fracture immunolabeling studies show that MIP(AQP0) localizes with fiber gap-junction plaques at their initial stage. When large junctional plaques are assembled, MIP(AQP0) mainly associates at the periphery of junctional domains, suggesting a potential role in the clustering and gathering of six-connexin hemichannels called connexons (Dunia et al., 1998).

The sequence of full-length cDNA of chick lens MIP(AQP0) has not been reported but a partial sequence of chick MIP(AQP0), mainly at the C-terminus, has been reported (Kodama et al., 1990). The lack of information on the full-length cDNA could be because this cDNA is highly GC rich (estimated at 75-80%), which leads to complicated secondary structures and low cloning efficiency. In the current study, by applying a PCR approach specific for a GC-rich sequence, we isolated, for the first time, a full-length clone of chick MIP(AQP0) from an embryonic lens cDNA library. Analysis of the amino acid sequence revealed information regarding the topology, molecular weight, and potential functional domains of the protein.

Previous studies have focused on the involvement of MIP(AQP0) in the formation of lens fiber gap junctions in adult lenses (Gruijters, 1989; Dunia et al., 1998) but the relationship between MIP(AQP0) and gap junctions during entire process of lens development has not been systematically characterized. The chicken offers an ideal model that permits an investigation of lens development at all stages in vivo, because the chick lens starts to develop at early stages relative to other species (Beebe and Piatigorsky, 1981). In our previous studies of the embryonic chick lens, although we could detect the interactions between two lens fiber connexins (Cx45.6 and Cx56), we failed to isolate a population of single connexons because of their association with another unknown protein. Instead, we were able to obtain a population of single connexons from the adult lens (Jiang and Goodenough, 1996). In this report, we identified MIP(AQP0) as the unknown protein associated with lens fiber connexins in embryonic chick lens. We found that MIP(AQP0) predominantly colocalized with gap junction plaques formed by Cx45.6 and Cx56 during the embryonic lens developmental process, but this association was limited to a narrow region close to the lens equator in the adult lens. The physical interactions were further evidenced by co-immunoprecipitation combined with immunoblot and protein pull-down analyses.

Materials and Methods

Materials

Fertilized, unincubated white leghorn chicken eggs were obtained from Ideal Poultry (Cameron, TX, USA) and incubated for the desired times in a humidified 37°C incubator. Adult chickens (1 month old) were obtained from a local farm and eye lenses were removed immediately after decapitation. Tissue-Tek compound was purchased from Miles Scientific (Naperville, IL, USA). Formaldehyde (16% stock solution) was from Electron Microscopy Sciences (Fort Washington, PA, USA). Alkaline-phosphatase-conjugated goat anti-rabbit IgG and goat anti-guinea pig IgG were from Promega (Madison, WI, USA). Octyl-β-D-glucopyranoside (8-Glu) and octyl-polyoxyethylene (8-POE) were from Biochem (Torrance, CA, USA). The Two-hybrid cDNA Library Construction kit, AdvanTage-GC 2 PCR kit and pCR2.1-TOPO TA Cloning kit were from CLONTECH (Palo Alto, CA, USA). QIAquick Gel Extraction kit and Ni-NTA Sepharose beads were from Qiagen (Valencia, CA, USA). Rhodamine-conjugated goat anti-rabbit IgG was from Boehringer Manneheim (Indianapolis, IN, USA). Vectorsheld® fluorescence mounting medium was from Vector Laboratories (Burlingame, CA, USA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH, USA). [^{35}S]-Methionine was from New England Nuclear (Boston, MA, USA). The Silver Staining kit was from Pharmacia (Peapack, NJ, USA). Bacterial expression vector pET-15b was from Novagen (Madison, WI, USA). All other chemicals were obtained from either Sigma (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Isolation of chick lens MIP(AQP0) cDNA clones

The chick lens cDNA library was constructed by using the Two-hybrid cDNA Library Construction kit according to the manufacturer's instructions. The cDNA was prepared from chick embryonic-day-11 lenses. The 5′-end primer (5′-GATACCCCACCAAACCCAAAAA-3′) was designed based on the sequence of the linker region flanking each cDNA, which was introduced when constructing the library. The 3′-end primer (5′-ACACACACGCACGCACCA-3′) was designed based on the partially known sequence of the 3′-untranslated region (UTR) of chick MIP(AQP0) cDNA (Kodama et al., 1990). Owing to the high GC content of the partial chick cDNA, the Advantage-GC 2 PCR kit was used to perform the PCR, with an annealing temperature of 56°C. After PCR amplification of over 30 cycles, multiple DNA fragments were obtained. The cDNA fraction from 700 bp to 1.2 kb was isolated using a 1% agarose gel, purified with the QIAquick Gel Extraction kit and then subcloned into vector pCR2.1-TOPO. From the information we obtained from both the molecular weight of chick MIP(AQP0) protein and the known sequences of MIP(AQP0) cDNA from other species, we deduced that the possible full-length chick MIP(AQP0) cDNA was 1.0-1.1 kb. The subclone with an insertion length of ~1050 bp was selected and sequenced (University of Texas Health Science Center at San Antonio, DNA Sequencing Core Facility). The sequence was confirmed by comparing it with the known 3′-end sequence of chick MIP(AQP0) cDNA, and with known sequences of full-length bovine (Gorin et al., 1984) (K02818) and partial frog (Austin et al., 1993) (X56970) MIP(AQP0) cDNAs.

Preparation and immunoaffinity purification of polyclonal antibodies, and generation of monoclonal antibody from hybridoma cells

Preparation and immunoaffinity purification of anti-Cx45.6 and anti-Cx56 antisera were performed as previously described (Jiang et al., 1994). Polyclonal anti-chick MIP(AQP0) antiserum raised from guinea pig and hybridoma cell lines used for generation of monoclonal anti-chick MIP(AQP0) antibody (Sas et al., 1985) were generous gifts from E. Tenbroke and R. Johnson (University of Minnesota). Hybridoma cells were cultured according to the protocols of Harlow and Lane (Harlow and Lane, 1999). Culture media containing the desired monoclonal antibody were obtained by collecting the supernatant after centrifuging at 500 ***g*** for 5 minutes. Using western blotting, the specificity of the antibody was confirmed by the detection of MIP(AQP0) protein in chick lens membrane lysate as previously characterized (Sas et al., 1985).

Immunohistochemistry

Chick embryos from various developmental periods were carefully dissected. Embryo lenses were removed and fixed in 2% formaldehyde (diluted from 16% stock in PBS) for 30 minutes at room temperature. Embryos were immersed in 1 M sucrose for 2 hours and then in Tissue-Tek compound for 5 minutes, and quickly frozen in

liquid nitrogen. Sagittal or coronal sections (10-20 μm) were then collected and prepared as described (Paul et al., 1991). For dual immunolabeling of MIP(AQP0) and Cx45.6, sections were first incubated in PBS for 5 minutes, then in blocking solution containing 2% normal goat serum, 2% fish-skin gelatin, 0.25% Triton X-100 and 1% bovine serum albumin (BSA) in PBS for 30 minutes, and finally with mixed antibodies: monoclonal anti-MIP(AQP0) antibody (1:2 dilution of hybridoma supernatant) mixed with affinity-purified anti-Cx45.6 antibody (1:500 dilution) in blocking solution overnight at 4°C. Sections were washed four times for 5 minutes each in PBS and then incubated with fluorescein-conjugated goat anti-mouse IgG against anti-MIP(AQP0) (1:500 dilution in blocking solution) for 2 hours at room temperature. Sections were washed four times for 5 minutes each in PBS and then incubated with rhodamine-conjugated goat anti-rabbit IgG against anti-Cx45.6 (1:500 dilution in blocking solution) for 2 hours at room temperature. After four washes in PBS for 5 minutes each, a drop of mounting medium was added before being covered by a glass cover slip. Control experiments included: labeling with each primary antibody individually to ensure that binding of one antibody was not sterically hindered by the other; labeling with mixed secondary antibodies only to detect any nonspecific cross-reactivity between secondary antibodies; and labeling with pre-immune antibody to determine any background bindings. The specimens were analysed using a confocal laser scanning microscope (Fluoview; Olympus Optical, Tokyo, Japan). FITC fluorescence was excited at 488 nm by an argon laser and rhodamine was excited at 543 nm with a HeNe-G laser. The emission filters used were BA505-525 for FITC and BA610 for rhodamine fluorescence.

Preparation of gap-junction-rich lens-fiber membrane

The gap junction-rich lens-fiber membranes were isolated as previously described (Jiang and Goodenough, 1996). Briefly, the whole chick lenses were lysed in the lysis buffer (5 mM Tris, pH 8.0 and 5 mM EDTA/EGTA) and crude membranes were pelleted at 100,000 ***g*** (Beckman SW60Ti rotor, 28,000 rpm) for 20 minutes at 4°C. Membranes were extracted first with 4 M urea, 5 mM Tris, pH 9.5 and 5 mM EDTA/EGTA, and followed with 20 mM NaOH before pelleted by centrifugation of 100,000 ***g*** for 45 minutes at 4°C. The membranes were washed with 5 mM Tris, pH 7.0, 2 mM EDTA/EGTA, 100 mM NaCl. Channel structures were solubilized with 1% octytl-polyoxyethylene (8-POE), 0.5% Triton X-100, 2% octyl-β-D-glucopyranoside (8-Glu) or 1% Nonidet P-40 for 10 minutes at room temperature (non-denaturing condition) or boiled for 3 minutes with 0.6% SDS (denaturing condition). The detergent-solubilized samples were separated from non-solubilized samples by centrifugation for 30 minutes at 100,000 ***g*** and supernatants were collected for further immunoprecipitation assay.

Metabolic labeling and immunoprecipitation

Intact lenses from embryonic-day-9 chickens were dissected into culture medium (medium 199 plus 10% fetal bovine serum) and metabolically labeled with [^{35}S]-methionine (0.5 mCi) for 3 hours (Musil et al., 1990; Jiang and Goodenough, 1996). The detergent-solubilized membranes were immunoprecipitated with affinity-purified anti-Cx45.6 or anti-Cx56 antibodies in the presence of 10 mM Hepes (pH 7.2) at 4°C overnight. Protein-A/Sepharose beads were then added and incubated for another 2 hours. The beads were washed three times with wash solution [10 mM Hepes, pH 7.2, 0.5% detergents (8-POE, Triton-X-100, Nonidet P-40 or 1% of 8-Glu)] plus 1% BSA. The immunoprecipitated samples were isolated from beads by boiling in SDS sample buffer for 5 minutes.

Immunoprecipitation of non-radioactive labeled samples was performed with antibodies [anti-MIP(AQP0) antiserum and affinity-purified antibodies against lens connexin] covalently conjugated to protein-A/Sepharose beads through a chemical cross linker, dimethyl pimelimidate as described (Harlow and Lane, 1988). On average, each lane on SDS-polyacrylamide (SDS-PAGE) contained an amount of the samples equivalent to one embryonic-day-11 lens or a quarter of a 1-month-old chick lens. Gap-junction-rich membrane preparation was immunoprecipitated with the conjugated immobilized antibodies in the presence of the wash solution as described above.

Preparation of a fusion protein containing six-histidine-tagged MIP(AQP0) C-terminus and analysis of protein pull-down

Based on the sequence of our cloned chick MIP(AQP0) cDNA, a DNA fragment encoding the C-terminus of MIP(AQP0) (amino acids 223-262) was produced by PCR using a chicken MIP(AQP0) cDNA clone as a template (sense primer, 5′-GGAATTCCATATGCTGTGTCCG-CGGGCG-3′; antisense primer, 5′-CCGCTCGAGCAGCCCCTGC-GTCTTC-3′). This fragment was inserted into the expression vector pET-15b. The recombinant fusion protein was expressed in *Escherichia coli*, induced by isopropyl-thio-β-D-galactoside and then isolated and purified with Ni-NTA beads, which bind the six-histidine epitope.

Total lens lysates obtained from day-10 chick embryos were first preincubated with Ni-NTA/Sepharose beads overnight at 4°C to eliminate any nonspecific binding to the beads. The supernatant fraction of the mixture was then saved and incubated with six-histidine-tagged MIP(AQP0) C-terminus fusion protein overnight at 4°C. Ni-NTA beads were then added to retain the fusion protein and its interacting protein(s). After 1 hour of incubation, the mixture was applied to a chromatography column and the flow-through fraction was collected. The beads were then washed with 20 mM imidazole solution containing 300 mM NaCl and 50 mM sodium phosphate, pH7.4, and the binding proteins were eluted using 200 mM imidazole solution. Protein components of both the eluted and flow-through fractions were then resolved on a SDS-PAGE and visualized by Coomassie Blue staining. The existence of Cx45.6 and Cx56 in each of the two fractions was further determined by western blot using corresponding specific antibodies.

SDS gel electrophoresis, fluorography, western blots, and silver staining

Immunoprecipitates were analysed on 12% SDS-PAGE gels. Gels loaded with immunoprecipitated [^{35}S]-labeled samples were processed for fluorography as described (Jiang et al., 1993). Western blots of lens lysates or immunoprecipitated samples were performed by probing with either anti-MIP(AQP0) antiserum (1:300 dilution) or affinity-purified anti-Cx45.6 or anti-Cx56 antibodies (1:500 dilution). Primary antibodies were detected with alkaline-phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) for anti-lens-connexin antibodies, and with alkaline-phosphatase-conjugated goat anti-guinea pig IgG (1:1000 dilution) for anti-MIP(AQP0) antiserum. The silver staining of proteins on SDS-PAGE gels was performed according to the manufacturer's instructions.

Results

Molecular cloning of chick lens MIP(AQP0) and sequence analysis

A PCR-based cloning strategy using a chick lens cDNA library yielded a 1063 bp cDNA (GenBank accession number AY078179) encoding a 262-amino acid protein with a predicted molecular weight of 28.1 kDa. The open reading frame is flanked by a 5′-UTR of 66 nucleotides and a 3′-UTR of 208 nucleotides (Fig. 1A). Comparison of this sequence at the nucleotide and amino acid levels with known databases (BLASTN and BLASTP, NCBI file servers) led to the identification of a chick ortholog of MIP and a member of

aquaporin family. The amino acid and nucleotide coding sequences of MIP(AQP0) are 79% and 72% identical, respectively, to those of bovine MIP(AQP0) (Gorin et al., 1984) (K02818). The alignment of amino acid sequences of chick and bovine MIP(AQP0) is shown in Fig. 1B. Chick MIP(AQP0), like its bovine counterpart, is a plasma-membrane protein with a high probability of having six transmembrane domains (Gorin et al., 1984; Drake et al., 2002).

Association of MIP(AQP0) with Cx45.6 and Cx56 in embryonic lenses

Biochemical studies with embryonic-day-9 chick lenses revealed that MIP(AQP0) was likely to form a complex with the lens-fiber connexins Cx45.6 and Cx56 (Fig. 2). Cultured embryonic chicken lenses were metabolically labeled and cell lysates were treated in the presence of SDS (denaturing condition) (Fig. 2, lanes 1 and 3) or 8-POE (non-denaturing condition) (Fig. 2, lanes 2 and 4), followed by immunoprecipitation with either affinity-purified anti-Cx45.6 (Fig. 2, lanes 1 and 2) or anti-Cx56 (Fig. 2, lanes 3 and 4) antibodies. Under the non-denaturing conditions, an additional protein band appeared (Fig. 2, arrowhead). This band has the same mobility on SDS-PAGE as MIP(AQP0) (Fig. 2, lane 5), which was revealed by the immunoblot of lens lysate using anti-MIP(AQP0) antiserum. Similar results were obtained for the samples treated with other types of nonionic detergents including Triton X-100, 8-Glu and Nonidet P-40 (data not shown) (Jiang and Goodenough, 1996).

A

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   1  TCTCTCGAGGATCTGAATTCGCGGCCGCGTCGACAGCGGGGCCGCGGCGGGCAGCGGGGCCGGGCC

  67  ATGCGGGAGCTGCGCTCGTCCTCCTTTTGGAGGGCCATCCTGGCCGAGTTCCTGGGCAGCCTCCTC
   1  M  R  E  L  R  S  S  S  F  W  R  A  I  L  A  E  F  L  G  S  L  L
 133  TACACCCTGCTGGGGCTGGGGGCTTCACTGCGCTGGGCCCCGGGCCCCCACGGGGTCCTGGGGTCC
  23  Y  T  L  L  G  L  G  A  S  L  R  W  A  P  G  P  H  G  V  L  G  S
 199  GCCTTGGCCTTCGGCCTGGCCCAAGCCACCCTGGTGCAGGCGCTGGGGCACGTCAGCGGAGGGCAC
  45  A  L  A  F  G  L  A  Q  A  T  L  V  Q  A  L  G  H  V  S  G  G  H
 265  ATCAACCCGGCCATCACGCTGGCCTTCCTGCTGGCCTCGCAGCTCTCCCTGCCCCGTGCCCTGGGC
  67  I  N  P  A  I  T  L  A  F  L  L  A  S  Q  L  S  L  P  R  A  L  G
 331  TACCTGCTGGCTCAGCTGCTGGGTGCCCTGGCGGGGGCCGGCGTCCTCTATGGGGTGACACCGGCC
  89  Y  L  L  A  Q  L  L  G  A  L  A  G  A  G  V  L  Y  G  V  T  P  A
 397  GCCGTGCGCGGCACGCTGGGCCTCAGTGCGCTGCACCCCAGCGTGGGTCCGGGCCAGGGCACGGTG
 111  A  V  R  G  T  L  G  L  S  A  L  H  P  S  V  G  P  G  Q  G  T  V
 463  GTGGAGCTGCTGCTGACGGCTCAGTTCATCCTCTGCGTCTTCGCCAGCTTCGACGACCGCCATGAC
 133  V  E  L  L  L  T  A  Q  F  I  L  C  V  F  A  S  F  D  D  R  H  D
 529  GGGCGCCCTGGCTCTGCTGCGCTGCCCGTTGGCTTCTCTCTCGCCCTCGGCCACCTCTTTGGGATC
 155  G  R  P  G  S  A  A  L  P  V  G  F  S  L  A  L  G  H  L  F  G  I
 595  CCATTCACTGGTGCTGGCATGAACCCCGCGCGGTCCTTTGCGCCCGCTGTCATCACCCGCAACTTC
 177  P  F  T  G  A  G  M  N  P  A  R  S  F  A  P  A  V  I  T  R  N  F
 661  ACCAACCACTGGGTGTTCTGGGCGGGTCCGCTGCTGGGCGCGGCGCTGGCGGCTCTGCTGTACGAG
 199  T  N  H  W  V  F  W  A  G  P  L  L  G  A  A  L  A  A  L  L  Y  E
 727  CTGGCGCTGTGTCCGCGGGCGCGCAGCATGGCGGAGCGCCTGGCCGTGCTGCGGGGGGAGCCGCCC
 221  L  A  L  C  P  R  A  R  S  M  A  E  R  L  A  V  L  R  G  E  P  P
 793  GCCGCCGCGCCGCCCCCCGAACCGCCGGCGGAACCGCTGGAGCTGAAGACGCAGGGGCTGTAG
 243  A  A  A  P  P  P  E  P  P  A  E  P  L  E  L  K  T  Q  G  L  *

 856  GGCCGCCGCCCCCCTCCATCCCCACCCCTCCCCTCACCCCCAAAGCCGGGGCCGCCTTTGCTGTCG
 922  GGGCGCCGATGCGGTGCGTGTGGTGCGTGCGTGTGTGTGCGGCGCGGCGGGAGTGCGGCGGCCACC
 988  GGCACCCAACGGGCGGCCCCCGCCGCCGCGGCGGGAGCTCCGTGTTCGGTGAATTAAACCTACGCT
1054  TTATTTTGGT
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B

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Chick:  1   MRELRSSSFWRAILAEFLGSLLYTLLGLGASLRWAPGPHGVLGSALAFGLAQATLVQALG 60
Bovine: 1   MWELRSASFWRAICAEFFASLFYVFFGLGASLRWAPGPLHVLQVALAFGLALATLVQAVG 60
                            (TM1)                          (TM2)

Chick:  61  HVSGGHINPAITLAFLLASQLSLPRALGYLLAQLLGALAGAGVLYGVTPAAVRGTLGLSA 120
Bovine: 61  HISGAHVNPAVTFAFLVGSQMSLLRAICYMVAQLLGAVAGAAVLYSVTPPAVRGNLALNT 120
                                          (TM3)

Chick:  121 LHPSVGPGQGTVVELLLTAQFILCVFASFDDRHDGRPGSAALPVGFSLALGHLFGIPFTG 180
Bovine: 121 LHPGVSVGQATIVEIFLTLQFVLCIFATYDERRNGRLGSVALAVGFSLTLGHLFGMYYTG 180
                            (TM4)                          (TM5)

Chick:  181 AGMNPARSFAPAVITRNFTNHWVFWAGPLLGAALAALLYELALCPRARSMAERLAVLRGE 240
Bovine: 181 AGMNPARSFAPAILTRNFTNHWVYWVGPVIGAGLGSLLYDFLLFPRLKSVSERLSILKGS 240
                                        (TM6)

Chick:  241 PPAAA-PPPEPPAEPLELKTQGL 262
Bovine: 241 RPSESNGQPEVTGEPVELKTQAL 263
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Fig. 1. Amino acid sequence of chick lens MIP(AQP0). (A) Sequence of cDNA clone of MIP(AQP0). A single uninterrupted open reading frame starting at nucleotide 67 and ending at nucleotide 885 (bold, underline), encoding a protein with a predicted molecular mass of 28.1 kDa. The derived amino acid sequence of MIP(AQP0) is shown in lower line. This sequence is registered in the GenBank database under the accession number AY078179. (B) The deduced amino acid sequence of chick MIP(AQP0) in one-letter code is aligned to the bovine lens MIP(AQP0) (K02818). Identical residues are shaded. Putative membrane-spanning domains (TM) are labeled and underlined.

Uniform colocalization of MIP(AQP0) with fiber connexins during early development and segregated distribution during late development

The association between chick lens fiber connexins and MIP(AQP0) at various stages of embryonic lens development was documented by dual immunolabeling of lens sections using two specific antibodies: monoclonal MIP(AQP0) antibody mixed with affinity-purified anti-Cx45.6 or anti-Cx56 antibody. The co-immunolabeling of MIP(AQP0) and lens-fiber connexins was performed using sagittal sections from various regions of embryonic-day-10 lenses including bow and core regions. Without exception, MIP(AQP0) was uniformly associated with all the junctional plaques formed by Cx45.6 in the lens fibers (Fig. 3A, A-F). The uniform colocalization was also observed between MIP(AQP0) and Cx56 (data not shown), consistent with the previous results that Cx45.6 and Cx56 coexist in the same junctional plaques (Jiang and Goodenough, 1996; Konig and Zampighi, 1995). The tissue sections from embryonic-day-20 lenses also manifest the colocalization between Cx45.6 and MIP(AQP0) in most areas of the lens including the bow region (Fig. 3B, A-C). However, this uniform colocalization appeared to segregate in the limited region around the center core nucleus of embryonic-day-20 lens (Fig. 3B, D-F). To confirm the colocalization patterns observed in embryonic-day-10 and -20 lenses, coronal lens sections were dual immunolabeled with anti-Cx45.6 and anti-MIP(AQP0) antibodies (Fig. 4). Consistent with the observations obtained from the dual immunostaining of sagittal sections, MIP(AQP0) and Cx45.6 were consistently

colocalized in the bow and core regions of embryonic-day-10 lens (Fig. 4A, A-F). In the embryonic-day-20 lens, although most Cx45.6 and MIP(AQP0) was colocalized around the bow region (Fig. 4B, A-C), this colocalization was segregated towards the core region of the lens (Fig. 4B, D-F). More interestingly, MIP(AQP0) was expressed predominantly on the short side of lens fiber, which separated the layers of lens fibers; Cx45.6, by contrast, was expressed preferentially on the long side of the lens fibers, which separated the cells within the same fiber layers (Fig. 4B, D-F).

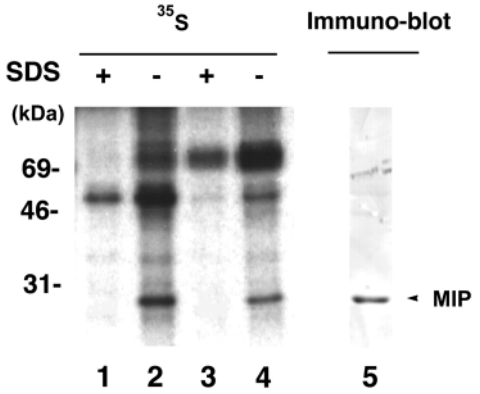

Fig. 2. Co-immunoprecipitation of MIP(AQP0) with embryonic lens fiber connexins. [^{35}S]-Methionine-labeled gap-junction-rich membranes isolated from embryonic-day-9 lenses were immunoprecipitated with affinity-purified anti-Cx45.6 (lanes 1 and 2) and anti-Cx56 (lanes 3 and 4) antibodies in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 0.6% SDS. Lens lysate was immunoblotted by anti-MIP(AQP0) antiserum (lane 5).

Interaction of MIP(AQP0) with lens fiber connexins in embryonic lenses

To verify the associations between MIP(AQP0) and lens-fiber connexins Cx45.6 and Cx56, an experimental approach was used based on our previous work (Jiang and Goodenough, 1996). In this assay, gap-junction-rich membrane prepared from embryonic-day-11 lenses was immunoprecipitated with immobilized affinity-purified anti-Cx45.6 or anti-Cx56 antibody, or anti-MIP(AQP0) antiserum under non-denaturing (with 8-POE) or denaturing (with SDS) conditions (Fig. 5). The resulting immunoprecipitates were then immunoblotted with anti-Cx45.6 (Fig. 5A) or anti-Cx56 (Fig. 5B) antibody, or anti-MIP(AQP0) antiserum (Fig. 5C). The result showed that Cx45.6, in addition to being present in the immunoprecipitates by anti-Cx45.6 antibody (Fig. 5A, lanes 1 and 2), was detected (Fig. 5A, arrowhead) in the immunoprecipitates of Cx56 (Fig. 5A, lane 3) and MIP(AQP0) (Fig. 5A, lane 5) in the absence of SDS. Similarly, Cx56 and MIP(AQP0) were present in the immunoprecipitates of Cx45.6/MIP(AQP0) and Cx45.6/Cx56, respectively. There was an additional band (Fig. 5A-G, *) in the immunoblot, which was likely to be the result of the cross-reactivity with immunoglobulin light chain protein (25 kDa) because this band also appeared in the samples treated with SDS. Moreover, this band was also detected when Cx45.6 antibody-conjugated beads alone were boiled in SDS and the precipitates blotted with anti-MIP(AQP0) antiserum (Fig. 5C, lane 7). To exclude the possibility that MIP(AQP0) might contaminate the co-immunoprecipitates

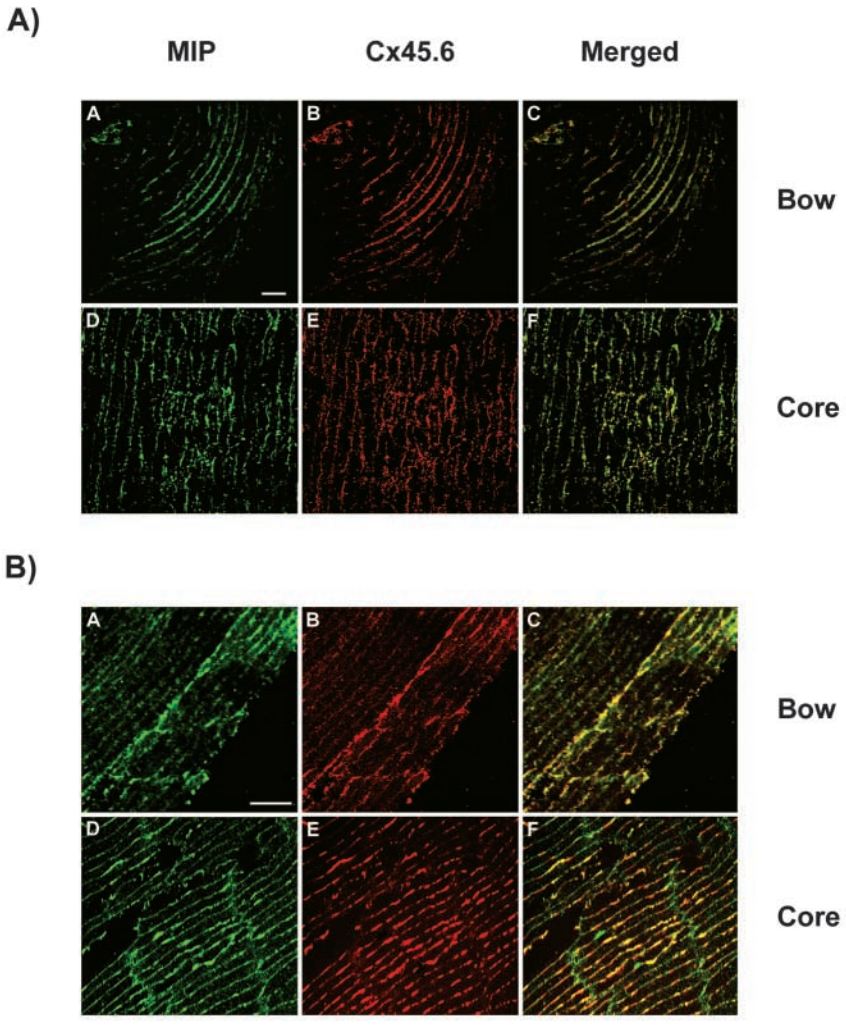

Fig. 3. Expression and colocalization of MIP(AQP0) and Cx45.6 detected by dual-immunostaining of sagittal sections of embryonic-day-10 and -20 lens. (A) Sagittal sections from bow (A-C) and core (D-F) regions of embryonic-day-10 chick lens were prepared and co-immunostained with antibodies specific for MIP(AQP0) and Cx45.6, and subsequently stained with fluorescein-conjugated goat anti-mouse IgG for MIP(AQP0) (A,D) and followed by rhodamine-conjugated goat anti-rabbit IgG for Cx45.6 (B,E). The corresponding images from the same regions were merged together to demonstrate the overlapping patterns between these two proteins (C,F). Scale bar, 20 μm. (B) Sagittal sections from embryonic-day-20 chick lens were similarly prepared and co-immunostained with anti-MIP(AQP0) monoclonal antibody (A,D) and affinity-purified anti-Cx45.6 (B,E) antibodies. The merged images were shown in (C,F). Scale bar, 20 μm.

owing to its abundance in the lens membranes, we silver stained the immunoprecipitates and supernatant derived from Cx45.6 affinity-purified antibody-conjugated beads (Fig. 5D). The pattern of the proteins separated by SDS-PAGE in supernatant of immunoprecipitated samples resembled those in total lens membrane preparation (Fig. 5D, lanes 1 and 2). However, immunoprecipitates resolved on the silver-stained gel showed bands of the same three sizes as those of Cx45.6, Cx56 and MIP (Fig. 5D, lane 3). Furthermore, the existence of MIP(AQP0) was confirmed by western blot using monoclonal anti-MIP(AQP0) antibody (Fig. 5D, lane 7). Non-existence of other protein was further confirmed by running a fivefold-overloaded sample (Fig. 5D, lane 4). No proteins were detected in the SDS-boiled precipitates of Cx45.6 antibody-conjugated beads without lysates (Fig. 5D, lane 5) or non-Cx45.6 antibody-conjugated beads in the presence of lysates (Fig. 5D, lane 6). The silver-staining experiments combined with the results from Fig. 2 provide evidence that the complex is probably formed by lens-fiber connexins and MIP(AQP0). Together, the interaction between Cx45.6 and Cx56 identified in this assay has confirmed our previous observation (Jiang and Goodenough, 1996). The new evidence indicated that MIP(AQP0) forms a complex with Cx45.6/Cx56 in embryonic lens fibers.

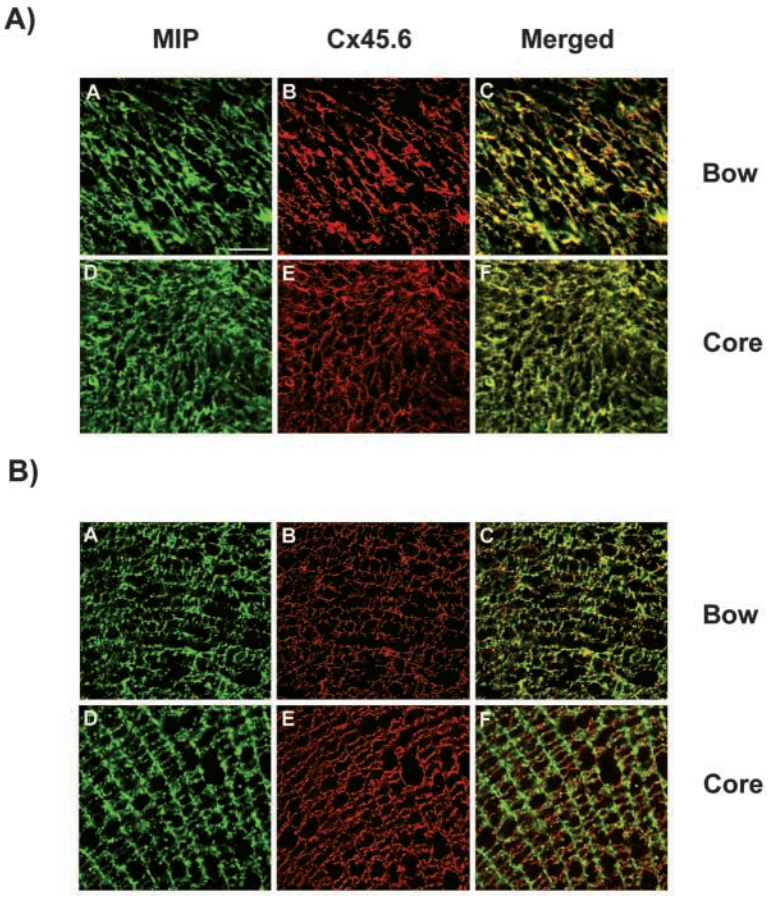

Fig. 4. Expression and colocalization of MIP(AQP0) and Cx45.6 detected by dual-immunostaining of coronal sections of embryonic-day-10 and -20 lens. (A) Coronal sections from bow (A-C) and core (D-F) regions of embryonic-day-10 chick lens were prepared and co-immunostained with antibodies specific for MIP(AQP0) and Cx45.6, and subsequently stained with fluorescein-conjugated goat anti-mouse IgG for MIP(AQP0) (A,D) and followed by rhodamine-conjugated goat anti-rabbit IgG for Cx45.6 (B,E). The corresponding images from the same regions were merged together to demonstrate the overlapping patterns between these two proteins (C,F). (B) Coronal sections from embryonic-day-20 chick lens were similarly prepared and co-immunostained with anti-MIP(AQP0) (A,D) and Cx45.6 (B,E) antibodies. The merged images were shown in (C,F). Scale bar, 20 μm.

C-terminus of MIP(AQP0) interacts with lens-fiber connexins

To confirm the specific interaction between MIP(AQP0) and lens connexins, the six-histidine fusion protein containing C-terminus of MIP(AQP0) was used to pull down proteins from lens lysates (whole protein profile shown in Fig. 6, lane 1). MIP(AQP0) is a six-membrane-span protein and the hydrophilic C-terminus is a potential domain sufficiently long for protein-protein interaction. The results showed that MIP(AQP0) C-terminus pulled down two proteins from lens lysate, with molecular weights similar to Cx45.6 and Cx56 (Fig. 6, lane 3). Western blots further confirmed the two proteins as Cx45.6 and Cx56 by using affinity-purified anti-Cx45.6 and anti-Cx56 antibodies (Fig. 6, lanes 4 and 7). Similarly, the existence of Cx45.6 and Cx56 was determined in the flow-through fraction (Fig. 6, lanes 5 and 8) and the original lysate (Fig. 6, lanes 6 and 9), respectively. No proteins were detected in the SDS-boiled precipitate of lysate by Ni-NTA beads without MIP(AQP0) C-terminus fusion protein (Fig. 6, lane 2). The retained Cx56 by MIP(AQP0) C-terminus appeared to be in a least phosphorylated form (Fig. 6, lanes 3 and 7). The preferred binding of MIP(AQP0) to the least phosphorylated form of Cx56 could be interpreted as follows: the complex might already be formed by fiber connexins and MIP(AQP0), in which the least-phosphorylated form of Cx56 might have lower affinity for endogenous MIP(AQP0) and readily competed off by added fusion protein containing C-terminus of MIP(AQP0). Alternatively, MIP(AQP0) C-terminus might prefer to bind the unphosphorylated form of Cx56, whereas other domains of MIP(AQP0) or Cx45.6 interact with the more phosphorylated forms of Cx56, as shown by the existence of multiple such forms of Cx56 in the co-immunoprecipitates (Fig. 5).

Transient association of MIP(AQP0) with lens-fiber connexins in adult lens

The relationship between MIP(AQP0) and lens-fiber connexins was examined in the lens sections of 1-month-old chicken (Fig. 7). MIP(AQP0) colocalized with Cx45.6 at the narrow bow region close to the lens equator (Fig. 7A-C). The results are consistent with the observation by Gruijters (Gruijters, 1989) that ovine MIP(AQP0) transiently associates with lens-fiber connexins close to the lens bow regions. Towards the central anterior (Fig. 7D-F) or posterior (Fig. 7G-I) regions of the lens, the colocalization gradually disappeared though some of the two proteins, Cx45.6 and MIP(AQP0), continued to localize

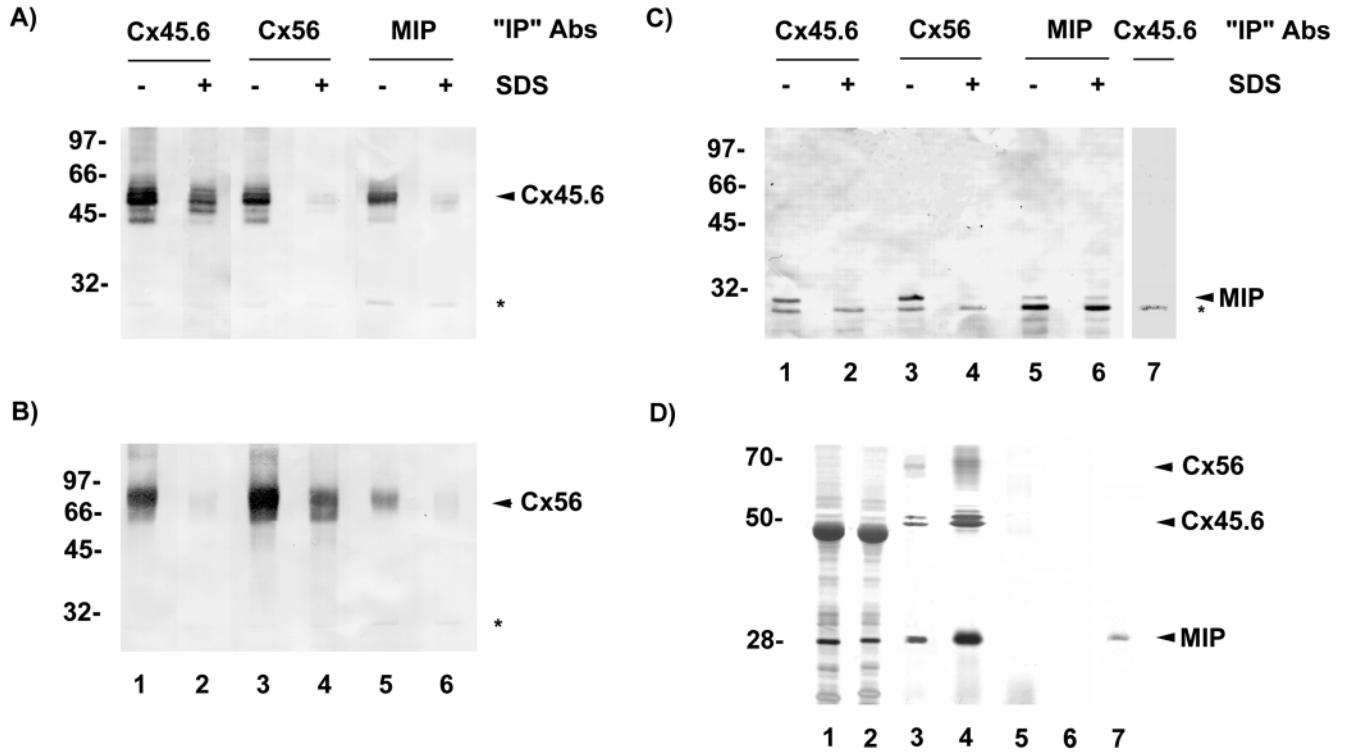

Fig. 5. Co-existence of MIP(AQP0) in the complex formed by Cx45.6/Cx56. Gap-junction-rich embryonic lens membranes were isolated from embryonic-day-11 lenses and immunoprecipitated with immobilized affinity-purified anti-Cx45.6 (A-C, lanes 1 and 2), anti-Cx56 antibodies (A-C, lanes 3 and 4) or anti-MIP(AQP0) antiserum (A-C, lanes 5 and 6) in the absence (A-C, lanes 1, 3 and 5) or presence (A-C, lanes 2, 4 and 6) of SDS. The resulting immunoprecipitates were immunoblotted with affinity-purified anti-Cx45.6 (A) or anti-Cx56 (B) antibodies, or with anti-MIP(AQP0) antiserum (C). The SDS-boiled precipitates of Cx45.6-antibody-conjugated beads alone were blotted by anti-MIP(AQP0) antiserum (C, lane 7). The cross-reacting immunoglobulin light chain was shown as (*). (D) Silver staining of the samples from membrane preparation of embryonic-day-10 lens (lane 1), supernatant of immunoprecipitation of lens membranes with anti-Cx45.6 antibody (lane 2), immunoprecipitates of lens membrane with anti-Cx45.6 antibody (lane 3), fivefold overloaded sample (lane 4), SDS-boiled precipitates with Cx45.6-antibody-conjugated beads in the absence of lysates (lane 5) and precipitates with non-Cx45.6-antibody-conjugated beads (lane 6) in the presence of lysates. The immunoprecipitates of lens membranes with anti-Cx45.6 antibody were immunoblotted by anti-MIP(AQP0) monoclonal antibody (lane 7).

with each other. Further into the lens nuclear core region, such colocalization was mostly disrupted because the MIP(AQP0) expression (Fig. 7J) and Cx45.6 expression (Fig. 7K) had different patterns in the merged image (Fig. 7L).

The lack of overall interactions was further shown by the above co-immunoprecipitation combined with immunoblot approaches (Fig. 8). The immunoprecipitates of Cx45.6 under non-denaturing conditions contained Cx56 but not MIP(AQP0) (Fig. 8A), and vice versa for Cx56 immunoprecipitates (Fig. 8B). Reciprocally, the MIP(AQP0) immunoprecipitates did not contain the lens-fiber connexins Cx45.6 and Cx56 (Fig. 8C). Thus, the disassociation of MIP(AQP0) and fiber connexins was supported by our biochemical assays in the adult lens.

Discussion

In this report, we isolated the full-length cDNA of chick lens MIP(AQP0) and systematically characterized the interactions between MIP(AQP0) and lens-fiber connexins in the embryo as well as in the adult lens development. There were two major findings. First, MIP(AQP0) uniformly colocalized with lens-fiber junctional plaques throughout the early stages of embryonic lens development. However, although there was still

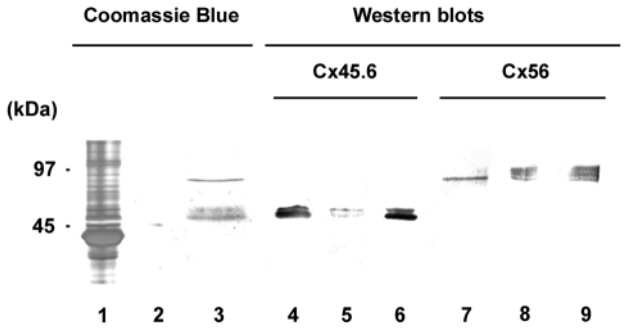

Fig. 6. C-terminus of MIP(AQP0) pulled down lens-fiber connexins from chick embryonic-day-10 lens lysates. Ni-NTA beads conjugated with six-histidine-tagged C-terminus of MIP(AQP0) were used to pull down proteins from lens lysates. Total lens lysates (lane 1) and retained fractions of lysate by Ni-NTA beads in the absence (lane 2) or presence (lane 3) of fusion protein were visualized by Coomassie Blue staining. The retained fraction of lysate by fusion-protein-conjugated-beads (lanes 4 and 7), the flow-through factions after extraction by fusion-protein/Ni-NTA beads (lanes 5 and 8) and total lens lysate samples (lanes 6 and 9) were immunoblotted with affinity-purified anti-Cx45.6 antibody (lanes 4-6) and anti-Cx56 antibody (lanes 7-9).

colocalization in most regions, the uniform co-distribution started to segregate around the nuclear core region in the later stages of embryonic development. In the adult lens, MIP(AQP0) associated only transiently with Cx45.6/Cx56 junctional plaques in the lens bow regions. Second, MIP(AQP0) and Cx45.6/Cx56 probably interact with each other in the embryonic lens and this interaction is most likely to occur at the C-terminal region of MIP(AQP0). The interaction was, however, negligible in the adult lens, even though there was a narrow zone of colocalization at the lens bow region.

Sequence and membrane topology analyses confirm that chick MIP(AQP0) is an ortholog of bovine MIP(AQP0) with more than 70% sequence identity and related to members of aquaporin family. Interestingly, in contrast to bovine MIP(AQP0), the nucleotide sequence of chick MIP(AQP0) is highly GC rich throughout the entire cDNA sequence. Previous unsuccessful attempts for cloning of chick MIP(AQP0) cDNA by standard PCR methods could be due to the fact that such GC-rich sequences possess stable secondary structure that would resist denaturation and prevent primer annealing (Chenchik et al., 1996). To circumvent this issue, we took advantage of a PCR approach specific for GC-rich sequences (Advantage-GC 2 PCR Kit, Clontech), which contains reagents that destabilize DNA secondary structure (Pomp and Medrano, 1991; Baskaran et al., 1996). The technical difficulties in the molecular cloning of chick MIP(AQP0) cDNA caused by the high GC content might explain why only fragments of MIP(AQP0) cDNA have ever been reported (Kodama et al., 1990).

It appears that Cx45.6 band from embryonic lenses was less intense under denaturing conditions in Fig. 5 than in Fig. 8 using adult lenses. Adult lens membranes are known to be more rigid, with tight packing and less fluidity than their embryonic counterparts (Anderson, 1983). When lens membrane is prepared, large portions of cell chunks may be formed that can only be efficiently dissolved by SDS. Thus, more Cx45.6 protein was observed under SDS-denaturing conditions at adult stage. The other differences could be due to the variation in membrane preparation and in the processes of sample loading. However, the difference will not compromise the validity of our observation because, in the adult lens, the absence of the MIP(AQP0) band was observed in both Cx45.6 and Cx56 immunoprecipitates, whereas no Cx45.6 or Cx56 was found in MIP(AQP0) immunoprecipitates.

Previous studies have mainly focused on the relationship between MIP(AQP0) and gap-junction plaques in adult lenses, which explains the lack of observation of the association of these two during lens development. Our studies with the adult chick lens are consistent in part with the observations in ovine lens that MIP(AQP0) associates with connexins in young lens fibers (Gruijters, 1989). The transient interactions between MIP(AQP0) and MP70 (ovine counterpart of Cx45.6 in chicken) have been observed in a narrow zone of the developing fibers but not in mature fibers. Another study using SDS fracture immunolabeling of the adult mouse lens has shown that, at the lens differentiation region, MIP(AQP0) localizes with Cx46/Cx50 (Dunia et al., 1998), which confirms our observation. When large junctions are assembled, MIP(AQP0) mainly associates in the periphery of the gap-junction domains. In addition, MIP(AQP0) has been observed to localize with fiber connexins to form an orthogonal lattice of repeating units in the central lens nuclear region. In our study, colocalization of Cx45.6/Cx56 with MIP(AQP0) in the adult lens nucleus was barely detectable. Most MIP(AQP0) expression formed a unique distribution pattern different from that of Cx45.6. This difference could be accounted by the difference in animal species or age, or by the limited resolution of confocal microscopy. Alternatively, MIP(AQP0) has been reported to undergo selective proteolysis at its C-terminus in

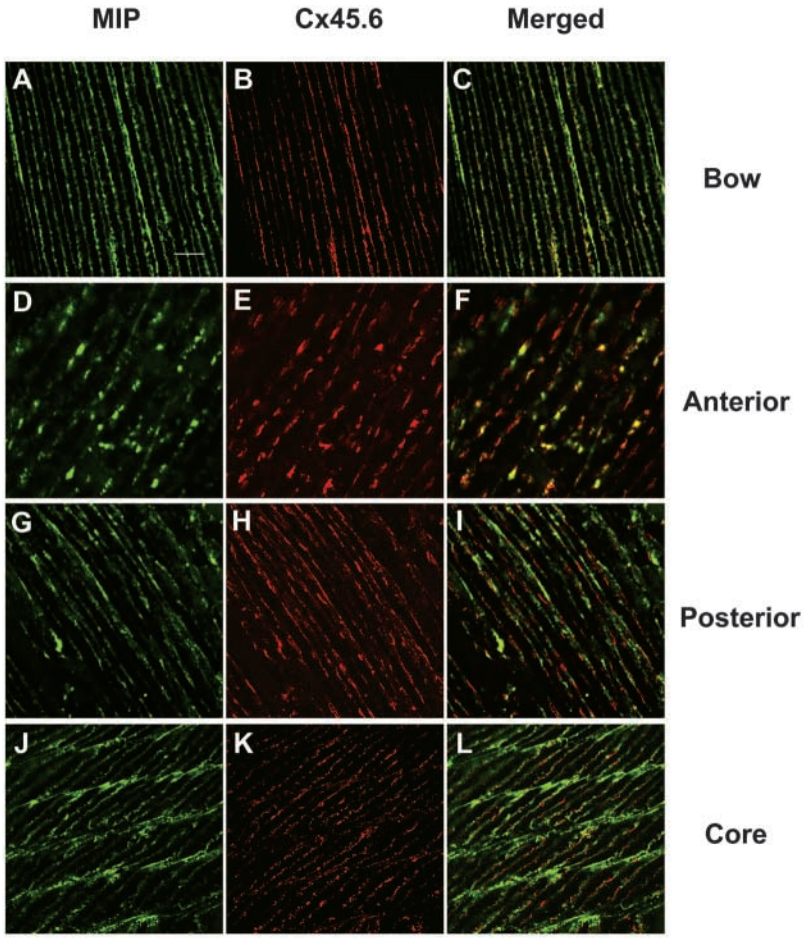

Fig. 7. Transient interactions of MIP(AQP0) with lens-fiber connexins at bow regions of adult lenses. Lens sections prepared from 1-month-old chicken were double labeled with monoclonal anti-MIP(AQP0) antibody and affinity-purified anti-Cx45.6 antibody, and subsequently labeled with fluorescein-conjugated goat anti-mouse IgG (A,D,G,J) for MIP(AQP0) and followed by rhodamine-conjugated goat anti-rabbit IgG for Cx45.6 (B,E,H,K). The corresponding images from the same regions were merged together to demonstrate the overlapping patterns between these two proteins (C,F,I,L). (A-C) A region at the lens bow regions; (D-F) a region at the anterior part of the lens fibers; (G-I) a region at the posterior part of the lens fibers; (J-L) a region at the center core of the lens fibers. Scale bar, 20 μm.

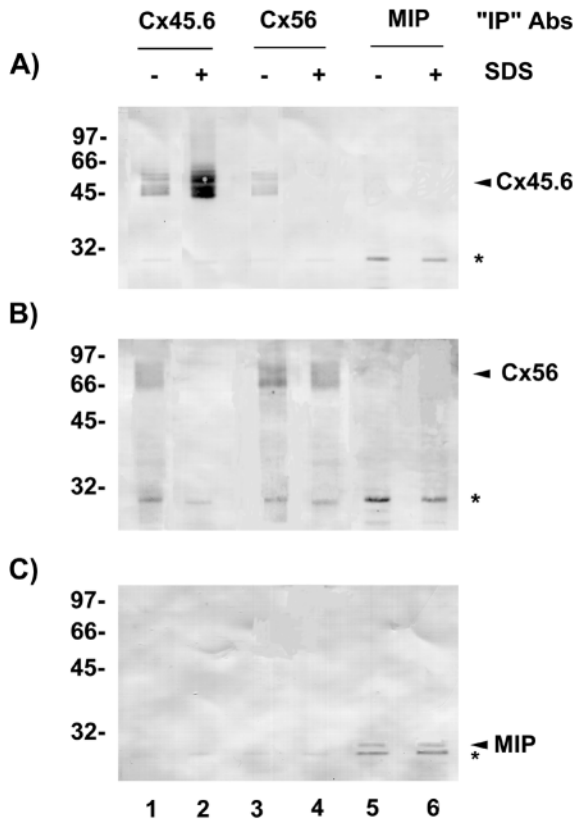

Fig. 8. MIP(AQP0) did not form a complex with lens-fiber connexins in adult chick lens. Gap-junction-rich lens membranes from postnatal 1-month-old chicken were prepared and immunoprecipitated with immobilized affinity-purified anti-Cx45.6 (lanes 1 and 2) or anti-Cx56 antibodies (lanes 3 and 4) antibody, or anti-MIP(AQP0) antiserum (lanes 5 and 6) in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of SDS. The resulting immunoprecipitates were immunoblotted with anti-Cx45.6 (A) or anti-Cx56 (B) antibodies, or anti-MIP(AQP0) antiserum (C). The cross-reacting immunoglobulin light chain is shown as (*).

the nucleus of aging human lenses (Takemoto et al., 1985). Because the monoclonal anti-MIP(AQP0) antibody used in our study is believed to target this region of the protein (Sas et al., 1985), the lack of colocalization between MIP(AQP0) and fiber connexins could be caused by the disappearance of the specific antigen of MIP(AQP0). Thus, the co-distribution and association patterns we observed could be interpreted as the interactions between full-length MIP(AQP0) and fiber connexins. However, we observed that the disappearance of association between MIP(AQP0) and Cx45.6 initiated at the late embryonic developmental stages and became dominant in the 1-month-old chicken, which occurs much earlier than the developmental periods when the cleavage of MIP(AQP0) is detected (Horwitz et al., 1979; Voorter et al., 1989). The association of MIP(AQP0) with lens-fiber connexins explains our previous unsuccessful attempts to isolate a population of single connexons formed by Cx45.6/Cx56 from embryonic chick lens lysate (Jiang and Goodenough, 1996). Instead, in that study, we successfully isolated pure connexons from adult lenses. This result agrees with our current observation in the interaction between MIP(AQP0) and lens-fiber connexins in embryonic lens and the lack of interactions in most adult lens-fiber cells.

There has been a controversy about whether MIP(AQP0) was the protein responsible for the formation of gap-junction channels (Sas et al., 1985; Kistler and Bullivant, 1987; Bok et al., 1982). In the early 1980s, Paul and Goodenough (Paul and Goodenough, 1983) showed that MIP(AQP0) only binds to non-junctional regions on the plasma membrane of bovine lens fibers. Electron microscopy of ultrathin frozen sections of rat lens (Fitzgerald et al., 1983) also shows that MIP(AQP0) distributes throughout the fiber-cell membrane, with no apparent distinction between junctional and non-junctional regions. With the identification of lens-fiber connexins and their further functional characterization (Kistler et al., 1985; Kistler et al., 1988; Varadaraj et al., 1999), it has become more clear that MIP(AQP0) is not the major channel-forming component of gap junctions. Although a definitive function for MIP(AQP0) in the lens has not been shown, mutation or deficiency of MIP(AQP0) causes cataracts and disruption of lens-fiber structures in the mouse (Shiels and Griffin, 1993; Shiels et al., 2000). During cataract development in the Nakano mouse lens, the decrease and final absence of MIP(AQP0) correlates with a decrease in gap junction structures (Tanaka et al., 1980). In one report, injection of antiserum against MIP(AQP0) into lentoids (cultured lens-fiber-like cells) resulted in the inhibition of cell coupling (Johnson et al., 1988). Recent observations made by Ahmed et al. (Ahmed et al., 2001) suggest that calmodulin, which is supposed to interact with C-terminal region of MIP(AQP0), might regulate the assembly of gap-junction channels in a cell culture. Lens membranes have abundant connexin expression at 72 hours and 84 hours of incubation, whereas MIP(AQP0) is absent or localized superficially at these developmental stages, indicting that MIP(AQP0) might not participate in the formation of gap junctions during early embryonic development (Yu and Jiang, unpublished). However, MIP(AQP0) appears to associate with gap junctions in young lens fibers and in the epithelium-to-fiber-cell differentiation zone, but not in the mature fibers (Gruijters, 1989). It is likely that MIP(AQP0) acts as a structural scaffold protein assisting later-formed lens-fiber gap-junction assembly during lens-fiber differentiation. When the mature gap junctions are formed, MIP(AQP0) appears to segregate from these junctions. MIP(AQP0) knockout mice have recently been generated (Shiels et al., 2001). In these mice, osmotic water permeability values of the lens were reduced and lenses developed opacities later in the animals' lives. More recent anatomical studies of MIP(AQP0) knockout mice report that lens fibers are disorganized and gap-junction-specialized structures were greatly reduced (Al-Ghoul et al., 2003), suggesting the close relationship between the presence of MIP(AQP0) and lens-fiber gap junctions. Future work will be directed at understanding the functional significance of these specific interactions during lens development.

We are grateful to E. Tenbroek and R. Johnson (University of Minnesota) for sharing anti-chick MIP(AQP0) antiserum and hybridoma cells for generation of monoclonal anti-chick MIP(AQP0) antibody, to X. Yin for providing anti-Cx45.6 antibody-conjugated beads, and to D. Adan-Rice and H. Barrus for technical assistance. This work was supported by grant EY-12085 from the National Institute of Health (J.X.J.) and a grant from the Welch Foundation (J.X.J.).

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