Developmental Regulation of the Direct Interaction between the Intracellular Loop of Connexin 45.6 and the C Terminus of Major Intrinsic Protein (Aquaporin-0)*

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The eye lens is dependent upon a network of gap junction-mediated intercellular communication to facilitate its homeostasis and development. Three gap junction-forming proteins are expressed in the lens of which two are in lens fibers, namely connexin (Cx) 45.6 and 56. Major intrinsic protein (MIP), also known as aquaporin-0 (AQP0), is the most abundant membrane protein in lens fibers. However, its role in the lens is not clear. Our previous studies show that MIP(AQP0) associates with gap junction plaques formed by Cx45.6 and Cx56 during the early stages of embryonic chick lens development but not in late embryonic and adult lenses. We report here that MIP(AQP0) directly interacts with Cx45.6 but not with Cx56. We further identified the intracellular loop of Cx45.6 as the interacting domain for the MIP(AQP0) C terminus. Surface plasmon resonance experiments indicated that the C-terminal domain of MIP(AQP0) interacts with two binding sites within the intracellular loop region of Cx45.6 with a $K_D^{(app)}$ of 7.5 and 10.3 μM, respectively. The $K_D^{(app)}$ for the full-length loop region is 7.7 μM. The cleavage at the intracellular loop of Cx45.6 was observed during lens development, and the C terminus of MIP(AQP0) did not interact with the loop-cleaved form of Cx45.6. Thus, the dissociation between these two proteins that occurs in the mature fibers of late lens development is likely caused by this cleavage. Finally this interaction had no impact on Cx45.6-mediated intercellular communication, suggesting that the Cx45.6-MIP(AQP0) interaction plays a novel unidentified role in lens fibers.

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The vertebrate eye lens is a valuable model system in the study of the function and regulation of gap junctions. The lens is an avascular organ formed by an anterior epithelial cell layer with highly differentiated fiber cells constituting the remaining lenticular mass. Mitotically active epithelial cells at the lens equator differentiate to give rise to the lens fibers, which lose intracellular organelles and accumulate high concentrations of soluble proteins known as crystallins (1). With the loss of cellular organelles, lens fibers lose the ability to support an active metabolism. In addition, there is no blood supply inside the lens. To maintain their metabolic activities and homeostasis, cells inside the lens depend fully on extensive networks of gap junction-mediated intercellular communications with the cells on the lens surface (2).

Gap junctions are channels connecting neighboring cells and allowing passage of small molecules (molecular mass, ≤1,000 Da) such as small metabolites, ions, and second messengers between the cytoplasm of two adjacent cells (3). The structural components of gap junctions are membrane proteins of a multigene family called connexins. All connexins have four conserved transmembrane domains and two extracellular loops, whereas their intracellular loops and C termini are the most variable regions (2). Three types of connexins are expressed in lens. Junctions between lens epithelial cells contain predominantly Cx43 in most of the animal species (4), whereas in human and rodent Cx50 and Cx46 are co-expressed predominantly in differentiated fiber cells (5, 6). Distinctly different physiological properties of gap junction channels formed by individual types of connexins confer unique functions in maintaining normal lens physiology (7). Targeted deletion of Cx46 results in nuclear cataracts (8) while deletion of Cx50 causes microphthalmia and pulverulent cataracts (9). This implies that Cx46 and Cx50 are likely to be differentially regulated by post-translational modifications and interaction with protein partners for the fulfillment of their unique roles in lens (10).

Another major fiber membrane protein, MIP, also known as aquaporin-0 (AQP0), belongs to a family of water transporter proteins including aquaporin-1 in kidney (11). MIP(AQP0) is the most abundant membrane protein in lens fibers, yet its function is still not clear. MIP(AQP0) does not form gap junction-like channels in the Xenopus oocyte pairing assay and baby hamster kidney cell transfection assay (12, 13). In addition, MIP(AQP0) has a very limited ability to transport water compared with other members of the family (14, 15), implying that the protein may have novel functions in lens. Previous reports have shown that MIP(AQP0) appears to transiently associate with differentiating fiber gap junction plaques in the adult lens (16), localizing predominantly at the periphery of junctional domains when large junctional plaques are assembled.

Association of non-connexin proteins with lens gap junctions has been reported in limited cases. The interaction has been documented between lens connexins and zonula occludens-1 (17). Our previous work has provided evidence that MIP(AQP0) associates with fiber gap junctions formed by Cx45.6 and Cx56, chick orthologs of Cx50 and Cx46, in early embryonic lenses.
and that these interactions involve the C terminus of MIP(AQP0) (18). The chick lens was chosen as our experimen-
tal system as it is very accessible for manipulation, allowing
intervention and study at all stages of lens development (19–
21). In the current report, we demonstrate for the first time
that the C terminus of MIP(AQP0) directly interacts with the
intracellular loop domain of Cx45.6. We found that the
dissociation of these two proteins in later lens development
was modulated by the cleavage at the intracellular loop domain
of Cx45.6. In addition, the specific interaction between
MIP(AQP0) and Cx45.6 was not observed to be involved in the
function of gap junction-mediated intercellular communication,
suggesting that the intervention of these two proteins plays a
yet unidentified, novel role in lens fibers.

EXPERIMENTAL PROCEDURES

Materials—Fertilized, unincubated chick eggs were obtained from
Ideal Poultry (Cameron, TX) and were incubated for the desired times
in a humidified 37 °C incubator. pET-15b bacterial expression vector was
generated by PCR using chick Cx45.6, Cx56, or MIP(AQP0) cDNA clone as
reagents were purchased from Invitrogen. Tracer molecules Lucifer
and the flow-through fraction was collected. The beads were then
added to washed 200 mM imidazole solution containing 300 mM NaCl and 50
mM sodium phosphate (pH 7.4). The binding proteins in the
mixture was then saved and incubated with the corresponding
His6-tagged fusion protein overnight at 4 °C. Ni-NTA beads were then
added to retain the fusion protein and its interacting protein(s). After a
1-h incubation the mixture was applied to a chromatography column,
and the flow-through fraction was collected. A single cysteine was
incubated to SDS-PAGE. Western blots of various
lysates or immunoprecipitated samples were performed by probing
with either monoclonal anti-MIP(AQP0) antibody (1:1 dilution) or
affinity-purified anti-Cx45.6 or anti-Cx56 antibodies (1:500 dilution).

Primary antibodies were detected with alkaline phosphatase-conju-
gated goat anti-mouse antibody-conjugated beads in the presence of 20 mM Na2B4O7 (pH 8.5) at
4 °C overnight. The beads were washed five times with wash solution
(20 mM Na2B4O7, pH 8.5, 0.5% Triton-X-100, 0.1% SDS, and 200 mM
sucrose). The immunoprecipitated samples were isolated from the
beads by washing in SDS sample buffer for 5 min and were then
subsequently SDS-PAGE.

SDS-PAGE, Western Blots, and Silver Staining—Immunoprecipi-
tates were analyzed on 12% SDS gels. Western blots of various
lysates or immunoprecipitated samples were performed by probing
with MIP(AQP0) antibody (1:1 dilution) or
affinity-purified anti-Cx45.6 or anti-Cx56 antibodies (1:500 dilution).

Primary antibodies were detected with alkaline phosphatase-conju-
gated goat anti-rabbit IgG (1:5,000 dilution) for anti-connexin anti-
odies and with alkaline phosphatase-conjugated goat anti-mouse
IgG (1:5,000 dilution) for anti-MIP(AQP0) antibody. The silver stain-
ing of proteins on SDS gels was performed according to the manufac-
turer’s instructions (Amersham Biosciences).

Peptide Synthesis and SPR—Three peptides (each ~25 amino acids
in length; >95% purity) were synthesized to cover the entire intracel-
ular loop domain of Cx45.6 with some overlapping sequences at the end
of each peptide (see Fig. 3A) by the University of Texas Health Science
Center at San Antonio Protein Core Facility. A single cysteine was
added to the N terminus of each peptide to facilitate the immobilization
of the peptide on a CM5 sensor chip (28). The SPR response is measured
in resonance units through the formation of disulfide bonds between the cysteine
residue and a derivatized carboxymethyl dextran-coated chip in a Bio-
core® 3000 system utilizing a thiol coupling kit (Biacore). GST or His6-
 fusion proteins containing the C terminus of MIP(AQP0) at the indi-
cated concentrations were injected over the sensor surface, and the
SPR

Interaction of MIP(AQP0) and Cx45.6
signals were recorded in real time utilizing Biacore Control software. A running buffer, HEPES-buffered saline containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P-20 with pH at either 7.4 mM NaCl, 3 mM EDTA, and 0.005% surfactant P-20 with pH at either 7.4 or 6.5, was used at a flow rate of 5 \mu l/min. Surfaces were activated with the injection of 10 \mu l of 6% guanidine hydrochloride in the buffer. For the competition studies, GST-MIP(AQP0) fusion protein was preincubated with the indicated concentration of peptides at room temperature for 5 min prior to injection. The affinity-purified Cx45.6 antibody against the intracellular loop domain was used as a positive control to demonstrate that the peptides had been successfully coupled to the chip. A scrambled peptide, referred to as the Random peptide, was immobilized at the same density to create a reference surface to reveal bulk flow changes that are caused by differences in the refractive index of the buffer and protein-containing solutions. SPR data were analyzed using BIAevaluation® software (Biacore) (29, 30).

Immunocytochemistry—CEF cells cultured on glass coverslips were co-infected with retroviruses expressing Cx45.6 and MIP(AQP0) as described above. For dual immunostaining of MIP(AQP0) and Cx45.6, cells were first fixed in 2% paraformaldehyde (from 16% stock) for 30 min at room temperature. After three washes with PBS, fixed cells were incubated in blocking solution containing 2% normal goat serum, 2% fish skin gelatin, 0.5% Triton X-100, and 1% bovine serum albumin in PBS for 30 min. Mixed antibodies of monoclonal anti-MIP(AQP0) antibody (1:2 dilution of hybridoma supernatant) and affinity-purified Cx45.6 antibody against its intracellular loop domain (1:500 dilution) in blocking solution were then added into culture plates and incubated overnight at 4 °C. Cells were washed four times for 5 min each in PBS and then incubated with fluorescein-conjugated goat anti-mouse IgG against anti-MIP(AQP0) antibody (1:500 dilution in blocking solution) for 2 h at room temperature. After four washes for 5 min each in PBS, cells were then incubated with rhodamine-conjugated goat anti-rabbit IgG against anti-Cx45.6 antibody (1:500 dilution in blocking solution) for 2 h at room temperature. After four washes in PBS for 5 min each, a coverslip was mounted onto a glass slide with a drop of mounting medium. The specimens were analyzed using a confocal laser scanning microscope (Fluoview, Olympus Optical, Tokyo, Japan). Fluorescein isothiocyanate fluorescence was excited at 488 nm by an argon laser, and rhodamine fluorescence was excited at 543 nm with a HeNe-Green laser. The emission filters used were BA505–525 for fluorescein isothiocyanate and BA610 for rhodamine fluorescence.

Scrape-loading Dye Transfer Assay for Gap Junction-mediated Intracellular Communication—The scrape-loading dye transfer assay was based on a published procedure (31). Briefly, briefly cells were scratched in the presence of two types of fluorescence dyes: RD (molecular mass, ~10 kDa) and either LY (molecular mass, 457 Da) or Alexa-488 (molecular mass, 570 Da). RD is too large to pass through gap junction channels and therefore serves as a tracer molecule for the cells originally receiving dyes. CEF cells with either Cx45.6 alone or Cx45.6 plus MIP(AQP0) retroviral infections were washed three times with Hanks’ balanced salt solution containing 1% bovine serum albumin for 5 min each. One percent RD and either 1% LY or 1% Alexa-488 dissolved in Hanks’ balanced salt solution were applied to the cell monolayers that were then washed lightly with a surgical blade. After incubation for 10 min, cells were washed with Hanks’ balanced salt solution three times and finally fixed in freshly made 2% paraformaldehyde (from 16% stock) for 30 min. The dye transfer results were examined with an Olympus fluorescence microscope (Tokyo, Japan) in which LY and Alexa-488 could be detected with the filter set for fluorescein, and RD could be detected with the filter set for rhodamine.

RESULTS

MIP(AQP0) Interacts with Cx45.6 but Not with Cx56—We have previously shown that MIP(AQP0), Cx45.6, and Cx56 form a complex on the plasma membrane of chick lens fiber cells in vivo and that the C terminus of MIP(AQP0) pulls down both Cx45.6 and Cx56 from embryonic day 10 lens membrane lysate (18). Because Cx45.6 and Cx56 always co-associate together in the lens (18, 32), it is experimentally unfeasible to utilize this system to determine which type(s) of these two fiber connexins interacts with MIP(AQP0). To address this issue, exogenous Cx45.6 or Cx56 was individually expressed in a connexin-deficient cell line, CEF cells. High expression was achieved by using recombinant retroviruses expressing either Cx45.6 or Cx56 (24, 25). A His$_6$-tagged fusion protein containing the C terminus of MIP(AQP0) was used in pull-down experiments with membrane lysates obtained from retrovirally infected cells either expressing Cx45.6 (Fig. 1A, lane 1) or Cx56 (Fig. 1A, lane 6). A single protein band observed in the retained fraction of the MIP(AQP0) C terminus pull-down from Cx45.6-expressing CEF cells (Fig. 1A, lane 2) was confirmed to be Cx45.6 by Western blot analysis (Fig. 1A, lane 3). No protein was pulled down by MIP(AQP0) C terminus from Cx56-expressing cells (Fig. 1A, lanes 7 and 8), resulting in a majority of Cx56 retained in the flow-through fraction (Fig. 1A, lane 9). Comparable levels of exogenous expression of Cx45.6 and Cx56 in CEF cell cultures were observed by Western blot analyses using specific antibodies (Fig. 1A, lanes 5 and 10). In addition to the binding of the C terminus, other cytoplasmic domains of MIP(AQP0), such as the N terminus and/or two intracellular loops, may potentially interact with Cx56. However, all three cytoplasmic domains have very limited lengths (10–20 amino acids in length), which renders such potential interactions sterically unfeasible (18, 33). Thus our results suggest that MIP(AQP0) does not directly interact with Cx56 and that the existence of Cx56 in co-immunoprecipitates of lens lysates is because of its association with Cx45.6 (18).

Co-immunoprecipitation was then performed to confirm the interaction between MIP(AQP0) and Cx56. Retroviruses containing either MIP(AQP0) cDNA (plus a short FLAG epitope at its C terminus) or Cx45.6 cDNA (without FLAG epitope) were co-infected into CEF cells. Bidirectional co-immunoprecipitation was conducted by subjecting membrane lysates to either anti-Cx45.6 antibody (Fig. 1B) or anti-FLAG antibody (Fig. 1C). When co-immunoprecipitated with anti-Cx45.6 antibody (Fig. 1B, lanes 2, 4, and 6), another protein with a molecular mass of ~28 kDa was also retained (Fig. 1B, lane 2) and confirmed to be MIP(AQP0) by Western blot analysis (Fig. 1B, lane 6). Similarly Cx45.6 was detected in co-immunoprecipitates of anti-FLAG antibody that was used to immunoprecipitate MIP(AQP0) (Fig. 1C, lanes 2, 4, and 6). Comparable expression levels of MIP(AQP0) and Cx45.6 were indicated by Western blot analyses of cell membrane lysates (Fig. 1, B and C, lanes 5 and 7). In addition to MIP(AQP0) and Cx45.6, two more unknown proteins were also co-immunoprecipitated by the anti-FLAG antibody (Fig. 1C, marked by asterisks) but not by the anti-Cx45.6 antibody. This may be explained by the possibility that MIP(AQP0) is able to bind to some endogenous proteins in CEF cells or that these proteins contain sequence(s) similar to that of FLAG epitope that may be recognized by the anti-FLAG antibody. Together our results establish a direct interaction between MIP(AQP0) and Cx45.6 both in vitro and in vivo.

Intracellular Loop Domain of Cx45.6 Pulls Down MIP(AQP0) from Embryonic Lens Lysate—To determine which portion(s) of Cx45.6 protein interacts with MIP(AQP0), fusion proteins containing cytoplasmic domains of Cx45.6 were prepared. We observed that when a His$_6$-tagged fusion protein containing the intracellular loop of Cx45.6 was incubated with lens membranes (total protein profile shown in Fig. 2, lane 1), a single protein with molecular mass of ~28 kDa was pulled down (Fig. 2, lane 3, arrow) and confirmed as MIP(AQP0) by Western blot analysis (Fig. 2, lane 4). Very limited amounts of MIP(AQP0) were detected in the flow-through fraction (Fig. 2, lane 5), whereas the protein was present in the total lens crude membranes (Fig. 2, lane 6). No proteins were detected in the precipitate of lens crude membrane by Ni-NTA beads lacking the Cx45.6 intracellular loop fusion protein (Fig. 2, lane 2). As a control, a His$_6$-tagged fusion protein containing the C-terminal domain of Cx56 was similarly used in pull-down assays, and no traces of MIP(AQP0) were observed in the retained fraction by either silver staining (Fig. 2, lane 7) or Western blot analysis.
ing eluted fractions were visualized by silver staining from Cx45.6- (lane 6) in the original CEF cell lysates was confirmed by Western blot analysis using the anti-Cx45.6 (lane 5) and anti-Cx56 (lane 7) antibodies. The co-expression of Cx45.6 and MIP(AQP0) (lane 1). Both MIP(AQP0) and Cx45.6 were retained from the lysates (lane 2) that were then detected on Western blots using anti-Cx45.6 (lane 4) and anti-MIP(AQP0) (lane 6) antibodies. The co-expression of Cx45.6 and MIP(AQP0) in the original CEF cell lysates was confirmed by Western blot analysis (lanes 5 and 7). The eluted fraction of the lysate from the control experiment with non-conjugated beads is shown in lane 3. Two proteins were retained in FLAG antibody co-immunoprecipitates (lane 2, marked with asterisks). IP, immunoprecipitation; Abs, antibodies.

FIG. 2. The intracellular loop domain of Cx45.6 pulls down MIP(AQP0) from chick embryonic lens lysate. Both His$_6$-tagged fusion proteins containing intracellular loop domain from either Cx45.6 or Cx56 were used in pull-down experiments using an embryonic day 10 lens membrane lysate (lane 1). The eluted fraction from the Cx45.6 intracellular loop pull-down was visualized by Coomassie Blue staining (lane 3), and the presence of MIP(AQP0) in the fraction was determined by Western blot analysis with the MIP(AQP0) antibody (lane 4). Similarly Western blots with the MIP(AQP0) antibody were also performed using the flow-through fraction (lane 5) and the lens lysate (lane 6). In the Cx56 loop pull-down experiment, the absence of MIP(AQP0) was shown by both silver staining (lane 7) and Western blot analysis with the MIP(AQP0) antibody (lane 8). Western blots of lens lysate with the MIP(AQP0) antibody are shown in lane 9. The eluted fraction of the lysate from the non-conjugated Ni-NTA beads is shown in lane 2. with the anti-MIP(AQP0) antibody (Fig. 2, lane 8). The presence of MIP(AQP0) in total lens crude membranes is shown in lane 9. Finally the C terminus of Cx45.6 failed to pull down MIP(AQP0) as indicated by the lack of MIP(AQP0) in the retained fraction by a fusion protein containing the C terminus of Cx45.6 (data not shown). The results suggest that the intracellular loop domain of Cx45.6 directly binds to MIP(AQP0).

The Front and Rear Portions of the Cytoplasmic Loop Domain of Cx45.6 Interact with the C Terminal of MIP(AQP0)—SPR was utilized to characterize the interaction between MIP(AQP0) and Cx45.6 (28). The full-length intracellular loop region of Cx45.6 was immobilized on one surface of a CM5 chip. An adjacent control surface was activated and blocked with cysteine. A GST fusion protein containing the MIP(AQP0) C terminus was injected over both surfaces. An SPR response was observed on the specific but not on the control surface, indicating a direct association between the full-length intracellular loop of Cx45.6 and the C terminus of MIP(AQP0) (Fig. 3B). To further map the binding site(s) of Cx45.6 on its loop domain, three overlapping peptides covering the entire intracellular loop of Cx45.6 and a control peptide were synthesized (Fig. 3A). The four peptides were coupled to adjacent surfaces of a sensor chip, and GST-MIP(AQP0) C terminus fusion protein was injected over the sensor surface (Fig. 3C). MIP(AQP0) interacted with both the Front and Rear peptides but did not interact with the Middle peptide or Random peptide. The signal changes observed on the Middle and Random peptide surfaces are referred to as “bulk flow changes,” which are caused by small differences in refractive index between the buffer and protein-containing solutions that cause a sharp rise in SPR signal at the start of the injection that is relatively flat for the duration of the injection and then abruptly returns to baseline at the stop of the injection (34). Bulk flow changes are not indicative of a specific association. When GST was passed over the same surfaces as a control, no significant binding was observed (Fig. 3D). This indicates that the interaction between the MIP(AQP0) C terminus and the Cx45.6 loop domain is specific.

During the differentiation process the cytosol of the lens fiber cells is gradually acidified from pH 7.0–7.4 at lens surface to pH 6.5 at the nucleus of the lens consisting of terminally differentiated fiber cells (35). To examine whether the intracellular pH decrease would affect the association between
MIP(AQP0) and Cx45.6, a binding experiment was performed at pH 6.5 (Fig. 3E). We observed that decreasing the buffer pH to 6.5 did not have any significant effect on the SPR signals elicited by the interactions between the MIP(AQP0) C terminus and the Front or Rear peptides, suggesting that the interactions are independent of the cytosol acidification occurring during fiber cell differentiation. Similarly low pH also had no effect on the interaction between Cx45.6 and MIP(AQP0) when half the amount of MIP(AQP0) fusion protein was used (data not shown).

To eliminate the possibility that the lack of SPR signal for the Middle peptide was caused by inefficient coupling or inactivation of the Middle peptide and to demonstrate the specificity of the interaction, an antibody specific for the intracellular loop domain of Cx45.6 at two different dilutions (Fig. 4, A, 1:50 dilutions, and B, 1:500 dilution) was injected over a sensor chip coupled with Front, Middle, Rear, and Random peptides. The anti-Cx45.6 loop antibody bound strongly to both the Front and Middle peptides but not to the Rear and Random peptides. This indicates that the Middle peptide was sufficiently coupled onto the sensor chip and that the immobilized peptide was in the configuration that was recognizable by the antibody. Our data also suggest that the antigenic determinants recognized by the anti-Cx45.6 loop antibody are located within the Front and Middle portions of the intracellular loop of Cx45.6. Together these results indicate that the C terminus of MIP(AQP0) directly interacts with both the Front and Rear portions of the intracellular loop domain of Cx45.6, and this specific interaction persists even at the low physiological pH existing in the differentiated lens fibers.

Two Binding Sites, Located at the Front and Rear Portions of Intracellular Loop Domain of Cx45.6, Interact with One Site on the C Terminus of MIP(AQP0)—Because MIP(AQP0) C terminus interacts with both Front and Rear peptides of the intracellular loop domain of Cx45.6, competition experiments were conducted to determine whether these two regions interact with the same site (sterically close) on MIP(AQP0) C terminus or two different sites (sterically independent). MIP(AQP0) C ter-
minus fusion protein was preincubated with either Front or Rear peptides followed by injection over the sensor chip coupled with the reciprocal peptide. The Rear peptide inhibited MIP(AQP0) C terminus binding to the Front peptide in a dose-dependent manner (Fig. 5A). Similarly binding between MIP(AQP0) C terminus and Rear peptide was also inhibited by the Front peptide in a dose-dependent manner (Fig. 5B). In both cases, the interaction between the fusion protein and the peptide was almost completely abolished by a 10-fold excess of the peptide competitor. As a control, preincubation with the Middle peptide did not decrease the interaction between MIP(AQP0) and Front or Rear peptides (data not shown). Thus, our data suggest that the interaction involves two binding sites on the cytoplasmic loop domain of Cx45.6 and one site (sterically close) on the C terminus of MIP(AQP0).

Various concentrations of His$_6$-tagged MIP(AQP0) C terminus fusion protein were injected over a surface coupled with full-length Cx45.6 cytoplasmic loop region, Front peptide, Rear peptide, or Random peptide. The signal from the Random peptide surface was subtracted from the raw signal obtained on each specific surface, and the resulting sensorgrams for MIP(AQP0) binding to full-length Cx45.6 loop (Fig. 6A), Front peptide (Fig. 6C), and Rear peptide (Fig. 6E) are displayed. Three independent sets of these experiments were performed, and the data points shown in Fig. 6B (full-length Cx45.6 loop), Fig. 6D (Front peptide), and Fig. 6F (Rear peptide) were obtained by averaging the maximal responses of the signals at each MIP(AQP0) concentration. The curves were then fit to a rectangular hyperbolic function by using BIADevaluation software (Biacore), and the $K_{D_{\text{app}}}$ was defined as the concentration of MIP(AQP0) to give half-maximal binding (29, 30). The $K_{D_{\text{app}}}$ of the interaction between MIP(AQP0) C terminus and Cx45.6 intracellular loop was found to be 7.7 nM, between MIP(AQP0) C terminus and the Front peptide was found to be 7.5 nM, and between MIP(AQP0) C terminus and Rear peptide was found to be 10.3 nM. These studies suggest that that the interaction between the intracellular loop of Cx45.6 and C terminus of MIP(AQP0) is saturable, specific, and of moderate affinity.

### Cleavage of Intracellular Loop Domain of Cx45.6 in the Differentiated Lens Fibers and Dissociation between the C Terminal Regions of MIP(AQP0) and Cx45.6—We have observed that interactions between MIP(AQP0) and Cx45.6 gradually disappear, directly correlating with late stages of lens development and the degree of lens cell differentiation into mature fibers (18). As shown in Fig. 3E, the lower pH of differentiated fibers of the lens nucleus failed to induce the dissociation of the interaction between MIP(AQP0) and Cx45.6. To understand the molecular mechanism that regulates the dissociation of these two proteins in differentiated lens fibers, we examined the profile of Cx45.6 expression during lens development by taking advantage of antibodies we have developed that are specific for the intracellular loop and the C-terminal regions of Cx45.6 (22, 23). Cx45.6 was gradually cleaved, and a predominant fragment appeared during chick
Starting from embryonic day 8, a cleaved form of Cx45.6 was detectable by anti-Cx45.6 antibody specifically against its C terminus (Fig. 7A, indicated by asterisk). The bands representing the cleaved form of Cx45.6 became stronger in later developmental stages (Fig. 7A, lanes 2–5), suggesting that the cleavage is directly associated with lens fiber differentiation and maturation. The cleavage was most likely to occur at the intracellular loop domain of Cx45.6 as indicated by the cleaved fragment with a molecular mass of ~40 kDa. Moreover this fragment was not detected by the anti-Cx45.6 antibody specifically against its intracellular loop region (Fig. 7A, lane 6). To determine whether such cleavage would abolish the interaction of Cx45.6 and MIP(AQP0), the His$_6$-tagged C terminus of MIP(AQP0) was used to perform pull-down experiments using embryonic day 18 lens crude membranes containing both the full-length Cx45.6 and the cleaved fragment. Western blots using the C terminus-specific anti-Cx45.6 antibody showed the presence of both the full-length and the fragment of Cx45.6 in lens membrane (Fig. 7B, lane 1) and the flow-through fraction (Fig. 7B, lane 4). Although the full-length Cx45.6 was retained in the eluted fraction, no fragment of Cx45.6 was detected (Fig. 7B, lane 3). Longer incubation with the anti-Cx45.6 antibody enhanced the signal of the Cx45.6 fragment in the flow-through fraction on the blot (Fig. 7B, lane 6), but again no trace of this fragment was observed in the eluted fraction (Fig. 7B, lane 5). None of the specific proteins were retained using non-conjugated Ni-NTA beads (Fig. 7B, lane 2). This suggests a potential mechanism for

**FIG. 6.** The interaction between Cx45.6 and the C terminus of MIP(AQP0) is saturable and specific. Interactions between a concentration series of MIP(AQP0) and full-length Cx45.6 cytoplasmic loop- (A and B), Front peptide- (C and D), Rear peptide- (E and F), or Random peptide-coupled surfaces were studied by SPR at pH 7.4. The response on the random surface was subtracted from the responses on each specific surface, and the MIP(AQP0) concentration-dependent SPR signals under each peptide are shown in A, C, and E, respectively. The maximal response at the end of each injection was plotted as function of the concentration of MIP(AQP0). The data were fit to a rectangular hyperbolic equation (B, D, and F), and the concentration of MIP(AQP0) to give half-maximal binding was taken as the $K_{D(app)}$ of the interaction. Plots shown represent averages from three independent experiments, and error bars indicate the standard error (n = 3). RU, resonance units.
The C terminus of MIP(AQP0) only binds to the full-length and not to the loop-cleaved form of Cx45.6. A, the development-associated cleavage of Cx45.6 at its intracellular loop domain from embryonic (Em) day 8 to postnatal (Post) day 150 (lanes 1–5). The cleaved form of Cx45.6 (marked by an asterisk) was detected by the anti-Cx45.6 antibody specifically against its C terminus but was not detected by the anti-Cx45.6 antibody against its intracellular loop domain (lane 6). B, His6-tagged C terminus of MIP(AQP0) was prepared to perform pull-down experiments using embryonic (Em) day 18 lens membrane lysate. Western blots probed with the antibody recognizing the Cx45.6 C terminus were performed with the cell lysate (lane 1), eluted fraction (lane 3), and flow-through fraction (lane 4). The binding between non-conjugated Ni-NTA beads and lysate is shown in lane 2. When the nitrocellulose membrane after Western blot transfer was incubated with the anti-Cx45.6 antibody at 4 °C overnight, the presence of the cleaved form of Cx45.6 in the flow-through fraction was indicated by the presence of a stronger band (lane 6, marked by an asterisk), whereas no trace of this fragment was detected in the eluted fraction (lane 5). Abs, antibodies.

**DISCUSSION**

This study, for the first time, demonstrates a direct physical interaction between MIP(AQP0), the most abundant lens fiber membrane protein, and gap junction-forming Cx45.6 and elucidates the underlying mechanism of the association/dissociation in differentiating and differentiated lens fibers. We systematically characterized the molecular properties and physiologically regulating mechanisms of the interaction, specifying the interaction as exclusively between MIP(AQP0) and Cx45.6. We further mapped two binding sites on the Front and Rear portions of the intracellular loop of Cx45.6 and a single site on the C terminus of MIP(AQP0), determined the apparent $K_d$ values of the interaction, and revealed the possible mechanism of the dissociation of the two proteins due to development-associated cleavage of the intracellular loop of Cx45.6.

Being the most abundant membrane protein in vertebrate lens fiber cells, the function of MIP(AQP0) has long been a matter of controversy. The prevalent theory so far recognizes MIP(AQP0) as the water-transporting protein for lens fibers (14) because protein sequence analysis categorizes MIP(AQP0) to the aquaporin water transporter family. However, among the aquaporins, MIP(AQP0) forms a water channel with distinctly low water permeability per molecule, some 40 times lower than that for aquaporin-1 (14, 15). Such inefficiency could be compensated for by the abundance of the protein expressed in lens fiber cells as it comprises more than half of all membrane proteins. Alternatively it has been hypothesized that MIP(AQP0) plays other unidentified functions in lens. Our study reported here implies that this interaction appears not to be related to the water transporting activity of MIP(AQP0) because Duchesne et al. (36) report that the C terminus of MIP(AQP0), the Cx45.6 binding domain, does not interfere with the transport activity of the protein. Studies conducted by Dunia et al. (37) and by our laboratory (18) demonstrate that the localization of MIP(AQP0) is closely correlated with newly formed gap junctional plaques that the narrow zone of the lens bow area where young fiber cells are actively differentiating, implying that the interaction between MIP(AQP0) and Cx45.6 may facilitate the assembly of the Cx45.6 into nascent gap junction plaques. The close relationship between MIP(AQP0) and lens gap junctions has been supported by observations made by Al-Ghoul et al. (38) in MIP(AQP0)-deficient mice that the size range of fiber gap junctions is significantly altered in both heterozygous and homozygous lenses and that the percentage of membrane areas in the midsegment of lens fibers specialized as gap junctional plaques is almost halved in homozygous lens as compared with those in wild-type lens. Earlier studies by Tanaka et al. (39) also show a correlation of the decrease and final absence of MIP(AQP0) with a decrease in gap junctional structures during cataract development. However, because co-expression of MIP(AQP0) and Cx45.6 in CEF cells did not cause any discernible alteration in Cx45.6-mediated intercellular coupling, it suggested that interaction between the two proteins is unlikely to be involved in the formation of functional gap junction channels. Fan et al. (40) recently show that γE-crystallin is recruited to the plasma membrane by interacting with MIP(AQP0) in mouse. MIP(AQP0) may have a similar role in the expression of Cx45.6 on the lens fiber membranes. Because MIP(AQP0) does not bind another fiber connexin, Cx56, this interaction may help to organize gap junctional plaques into Cx45.6-rich subcompartments, which would then contribute to the unique functions of this connexin in promoting cell differentiation and lens development (9). It will be interesting to further characterize the potential regulatory effect of MIP(AQP0) on Cx45.6-forming gap junctions and on lens physiology.

In this study, we observed that Cx45.6 was gradually cleaved during lens development. The cleavage site(s) was most likely located in the intracellular loop domain based on the mobility
of the cleaved form of Cx45.6 as resolved by SDS-PAGE as well as on the lack of detection of the cleaved fragment by anti-Cx45.6 antibody specifically against the intracellular loop region. Furthermore the antigenic determinants of the antibody against the loop domain determined by SPR experiments are located in both the Front and Middle peptides but not in the
and causes the dissociation and gradual separation of the two proteins until in differentiated mature fibers of lens nucleus where most of the interaction is abolished. It is of interest that the detailed structures of the Cx45.6 loop domain be resolved to further understand the molecular properties and function of the association between MIP(AQP0) and Cx45.6.

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Developmental Regulation of the Direct Interaction between the Intracellular Loop of Connexin 45.6 and the C Terminus of Major Intrinsic Protein (Aquaporin-0)
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