Amino Acid Residue Val\textsuperscript{362} Plays a Critical Role in Maintaining the Structure of C Terminus of Connexin 50 and in Lens Epithelial-fiber Differentiation\textsuperscript{*}

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We have previously shown that connexin (Cx) 50, unlike the other two lens connexins, Cx43 and Cx46, promotes chicken lens epithelial-fiber differentiation in a channel-independent manner. Here, we show that deletion of the PEST motif at the C terminus (CT) domain of Cx50 attenuates the stimulatory effect of Cx50 on lens fiber differentiation. Valine 362, a residue located within the PEST domain, is functionally involved. The structure of the Cx50 CT predicted by molecular modeling revealed four \( \alpha \)-helices and Val\textsuperscript{362} was found to be located in the middle of the 3rd helix. Replacement of Val\textsuperscript{362} with amino acid residues that disrupt the \( \alpha \)-helical structure predicted by molecular modeling, such as arginine, glutamate, or phenylalanine, attenuated the stimulatory effects of Cx50 on lens differentiation, whereas replacement with threonine, isoleucine, leucine, or proline, which maintain the structure preserved the function of Cx50. Circular dichroism (CD) studies supported the structural predictions and showed that the substitution with Glu, but not Thr or Pro, disrupted the \( \alpha \)-helix, which appears to be the structural feature important for lens epithelial-fiber differentiation. Together, our results suggest that Val\textsuperscript{362} is important for maintaining the helical structure and is crucial for the role of Cx50 in promoting lens epithelial-fiber differentiation.

The vertebrate eye lens is an avascular organ consisting of an anterior epithelial cell layer and highly differentiated fiber cells in the center. Mitotically active epithelial cells at the lens equator differentiate into the lens fiber cells, which lose major intracellular organelles and accumulate high concentrations of soluble crystallins (1). The loss of cellular organelles makes the fiber cells become incapable of supporting an active cellular metabolism. Hence, the fiber cells depend upon extensive networks of gap junction communications and transport system to direct microcirculation to support their metabolic needs and homeostasis (2).

Gap junctions are membrane channels that connect neighboring cells and allow the passage of small molecules (\( M_\text{r} \) < 1,000), such as small metabolites, ions, and second messengers. This type of cell-cell coupling is essential for normal cell and tissue functions. The structural components of gap junctions are membrane proteins called connexins. Connexins have four conserved transmembrane domains with the most variable cytoplasmic C terminus (CT)\textsuperscript{2} domain (3). Three types of connexins are expressed in the lens organ, among which Cx43 is predominately expressed in lens epithelial cells. During the transition process into fibers, the expression level of Cx43 is decreased and eventually replaced by two fiber connexins, Cx50 and Cx46. Each connexin forms channels with distinctive physiological properties of gating, permeation, and selective interaction with other connexins (4).

Intercellular communication through gap junction has been demonstrated to regulate vertebrate development, where “communication compartments” were used to describe the coupled cells sharing the same fate (5). In the lens, the importance of gap junctions and connexins was demonstrated by knock-out mouse models. Both Cx50- and Cx46-deficient mice develop cataracts. In addition, ablation of Cx50 results in decreased lens size, leading to microphthalmia (6–8). The retardation in lens growth caused by deficiency of Cx50 is likely due to the attenuation of lens epithelial-fiber differentiation (9). Concurring with in vivo observations, we have previously shown that overexpression of Cx50, but not other lens connexins, significantly stimulates lens epithelial-fiber differentiation in lens primary cell culture (10). Moreover, the CT domain of Cx50 is sufficient to promote lens fiber differentiation, suggesting the importance of this region (11).

In this study, we identified a critical amino acid residue, Val\textsuperscript{362} within the CT domain of Cx50 that is functionally involved in lens epithelial-fiber differentiation. Furthermore, we showed that this amino acid residue is an important component in maintaining an \( \alpha \)-helical structure, and this structural feature appears to be indispensable for the role of Cx50 in lens epithelial-fiber differentiation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fertilized, unincubated white leghorn chicken eggs were purchased from Ideal Poultry (Cameron, TX) and incubated for 10–11 days in a humidified 37 °C egg incubator. Anti-Cx50 antibody was generated as previously described (12). Anti-CP49 antibody was a generous gift from Dr. Paul Fitzgerald at the University of California (Davis, CA); anti-AQP0 polyclonal antibody was generated from rabbit against chicken AQP0 C terminus (223–262 amino acids). Rhodamine-conjugated goat anti-rabbit IgG and chemiluminescence kit (ECL) was purchased from GE Healthcare (Piscataway, NJ); paraform-

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\textsuperscript{2} The abbreviations used are: CT, C terminus; Cx, connexin; LY, Lucifer yellow; RD, rhodamine dextran; TFE, 2,2,2-trifluoroethanol; AQP0, aquaporin-0.
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TABLE 1
The primers used for site-directed mutagenesis studies

| Mutants   | Sense primer (5’–3’) | Antisense primer (5’–3’) |
|-----------|----------------------|------------------------|
| E328A + E331A | GACAACGAGGAGGGAGGACGGACGGAGAAAGTAAAGG | GACTTACCTTCTCCTCCGAGTCCGAGGCTTCTCCTCTTTTC |
| A348P     | GAAAGACGAGGGAGGAGGACGGAGAAAGTAAAGG | GACTTACCTTCTCCTCCGAGTCCGAGGCTTCTCCTCTTTC |
| V362E     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| P370A     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362P     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362R     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362K     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362L     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362I     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |
| V362F     | GAAAGACGAGGGAGGAGGAGGAGGAGAAAGTAAAGG | CACCTCCTGCACTCCCTCTCTCTCTCTCTCTCTCTCTTTC |

Preparation of Recombinant Retroviral Constructs Encoding Cx50 and Cx50 Mutants and Generation of High-titer Retroviruses—Retroviral constructs and high-titer retroviruses were prepared based on the protocol described previously (13, 14). Briefly, a cDNA fragment containing chicken Cx50 was generated by PCR and constructed into the retroviral vector RCAS(A). Cx50 single or multiple site mutants were generated with the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions with the primers shown in Table 1. PCR primers were synthesized and constructs were sequenced at the University of Texas Health Science Center at San Antonio DNA Core Facility.

Primary Chicken Lens Cell Culture—Primary chicken lens cell cultures were prepared as described (11). Briefly, lenses from 10–11-day-old chicken embryos were dissected, rinsed with TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 5 mM glucose, and 25 mM Tris (pH 7.4)), and digested with 0.1% trypsin at 37 °C for 30 min. Lens cells were obtained by breaking the lens apart and collecting them in M199 medium with 10% fetal bovine serum. Living cells were then counted and seeded at 3 × 105 cells per well of a 12-well culture plate. The next day after the primary culture was seeded, retroviruses expressing wild-type and mutated forms of Cx50 were added to primary lens cell cultures. The cultures were incubated at 37 °C, 5% CO2 and fed every other day. At the start of culturing, only monolayer lens epithelial cells proliferated on the culture plates. After 3–4 days, lens epithelial cells reached confluency and differentiated to form fiber-like “lentoid” structures. The number of lentoid was counted everyday during the culturing.

Immunofluorescence and Confocal Laser Microscopy—For immunolabeling of AQP0 expressed in the primary lens cell culture, a glass coverslip was placed into each well of a 12-well plate before seeding the cell. After 6–8 days of culturing, cells were fixed with 2% paraformaldehyde for 30 min and then incubated with blocking solution (2% goat serum, 2% fish skin gelatin, 0.25% Triton X-100, and 1% bovine serum albumin in Hanks’ balanced salt solution) for another 30 min. AQP0 was labeled with anti-AQP0 polyclonal antibody (1:200) followed by rhodamine-conjugated anti-rabbit IgG (1:400). The sections were mounted on a glass coverslip using Vectashield. The specimens were analyzed under a confocal laser scanning microscope (Fluoview; Olympus Optical, Tokyo, Japan). Image acquisition parameters were maintained consistent for each sample. Representative fluorescent images from random regions within each culture well were taken and the fluorescence signals of the AQP0 staining area versus the whole image area were determined using NIH ImageJ software (10). Ten to fifteen images for each sample were used to examine the AQP0 expression per measurement.

Sequence Analysis and Molecular Modeling of Cx50 CT—Multiple rounds of Psi-BLAST search in the NCBI data base were performed to identify the possible sequence homology. To construct a structural model for Cx50 CT based on human cyclin A2 (PDB code 3bht) as a template, the SWISS-MODEL online server (37) was used. The sequence alignment between Cx50 CT and a part of cyclin A (Ser171–Val630) was performed by the T-COFFEE program (38) which was then subjected to alignment mode modeling in SWISS-MODEL. Energy minimization of this Cx50 CT model was performed with GROMOS96 in DeepView software (39). No clashes within the individual subunits or at the subunit interfaces have been observed before or after energy minimization. To further confirm the structural information from the molecular modeling methods, other secondary prediction methods including SOPM (self-optimized method) were utilized to predict the Cx50 CT secondary structure solely based on the composition of amino acids (15). In addition, Profile Hidden Markov Models (HHpred program (16)) were also employed to predict the possible secondary structure of Cx50 CT by scoring pairs of aligned secondary structure states in a way analogous to the classical amino acid substitution matrices.

Scrape Loading Dye Transfer Assay and Fluorescence Microscopy—Chicken embryonic fibroblast cells were grown to confluence to maximize cell–cell contact. Scrape loading dye transfer was performed based on a modified protocol (17). Briefly, cells were scratched in the presence of two fluorescent dyes: LY (457 Da), which can pass through gap junction channels, and RD (10 kDa), which is too large to pass through gap junction channels. The presence of LY indicates those cells that participate in gap junction-mediated communication and RD serves as a tracer dye for cells originally receiving the dye. Cells were washed three times with Hanks’ balanced salt solution plus 1% bovine serum albumin for 5 min each and then a mix-
ture solution containing 1% LY, 1% RD in phosphate-buffered saline was applied and the plates were scraped lightly with a surgical blade. After 15 min of incubation with the dyes, the cells were washed with Hanks’ balanced salt solution three times, washed twice with phosphate-buffered saline, and then fixed in fresh 2% paraformaldehyde for 30 min. Dye transfer results were examined using a fluorescence microscope (Zeiss), where LY and RD could be detected by using fluorescein and RD filters, respectively. Image acquisition was kept consistent for all measurements and no threshold adjustments were used. Using RD staining as the reference for the original dye-loaded cells, the extent of dye transfer was measured as the distance from the center of the scrape line to the farthest extent of RD-or LY-stained cells with the appropriate scale bar. At least five images per condition tested with six measurements per image were used to assess the degree of dye transfer.

**SDS-PAGE, Fluorography, and Western Blotting**—Cultured cells were collected in lysis buffer (5 mM Tris and 5 mM EDTA/EGTA, pH 8.0) and then ruptured by passing through a 26 1/2-gauge needle multiple times. Lysates were centrifuged for 3 min at 1,000 g to remove cellular and nuclear debris. The supernatant was then centrifuged at 100,000 x g for 30 min (SW 60 Ti rotor, Beckman). The pellet was resuspended in lysis buffer and boiled in 0.6% SDS for 3 min. Lysates were analyzed by probing with anti-AQP0 (1:1,000), anti-CP49 (1:1,000), anti-AQP1 (1:1,000), anti-β-actin (1:5,000). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) or anti-mouse IgG (1:10,000) using chemiluminescence reagent kit (ECL).

**Circular Dichroism Spectroscopy**—Circular dichroism spectra were acquired in the far UV (185–250 nm) on a Jasco-815 CD spectropolarimeter at 6 °C using a 1-cm path length quartz cuvette. All spectra presented in graphs were accumulated and averaged from 5 scans. The peptides containing the second and third helices were generated with a tyrosine residue added to the C termini of each peptide to facilitate concentration determination (Table 2). The capability of the peptide to form a helical structure was evaluated by quantifying secondary structures using CD spectroscopy in the presence of varying concentrations of 2,2,2-trifluoroethanol (TFE) in 1 mM sodium phosphate buffer (pH 4.2, 7.2, or 8.8). Peptide concentration was maintained at 4 μM or 0.16 mg/ml. The secondary structure contents of the peptide were calculated using an online server DICHROWEB, which integrates SELCON, CDSSTR, and K2D analysis algorithms (18).

**Statistical Analysis**—Data were analyzed with one-way analysis of variance and Newman-Keuls multiple comparison test along with GraphPad Prism software (GraphPad Software, La Jolla, CA). Data are presented as the mean ± S.E. of at least three measurements. Asterisks represent the degree of significance in comparison with controls (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**RESULTS**

**Cx50 PEST Motif Is Functionally Involved in Lens Fiber Cell Differentiation**—We have previously shown that the CT domain of Cx50 is involved in lens epithelial-fiber differentiation (11). Several residues within a putative PEST motif (rich in amino acids Pro, Glu, Ser, and Thr) of Cx50 CT that we have previously identified are associated with Cx50 phosphorylation, protein stability, and caspase 3 cleavage (19, 20). To test if the removal of this motif has any impact on the role of Cx50 in lens fiber differentiation, a truncated form of Cx50 without the PEST motif (ΔPEST-Cx50) was generated (Fig. 1A). The expression of exogenous Cx50 and ΔPEST-Cx50 was achieved by infecting primary lens cells with recombinant retroviruses. Pulse-chase experiments showed that deletion of the PEST domain did not affect Cx50 protein turnover and stability. The number of lentoids, which are an indication of lens epithelial-fiber differentiation, was quantified each day during 8 days of culturing. The result showed that deletion of the PEST domain (ΔPEST-Cx50) significantly abolished the effect of Cx50 on promoting lens fiber differentiation as compared with wild-type Cx50 (Fig. 1B). The attenuated effect due to PEST motif deletion was further confirmed by the reduced expression level of AQP0, a marker for lens fiber differentiation by quantifying fluorescence intensity of immuno-labeled AQP0 (11, 21) (Fig. 1C). Within this PEST motif, we have shown that Ser368 is a caspase-3 cleavage site (20). Ser364 and Ser371 are phosphorylated residues (19) and phosphorylation of Ser364 regulates Cx50 turnover and caspase 3-mediated degradation (20). We generated single (E368A, E368D, S371A, S371D), double (S364A/S371A), and triple (S364A/E368A/S371A) mutants of these three residues by site-directed mutagenesis. However, none of the mutants compromised the influence of Cx50 on promoting lens fiber differentiation as indicated by the level of AQP0 expression (Fig. 1D).

Val362 within the PEST Domain Is a Critical Residue for the Role of Cx50 in the Lens Fiber Differentiation—To identify possible amino acid residue(s) of Cx50 involved in lens fiber differentiation, a multiple Psi-BLAST search in the NCBI data base was performed. Five highly conserved residues (Glu328, Glu331, Ala348, Val362, and Pro370) were identified between sequences of Cx50 and cyclin A. Among these residues, only two, Val362 and Pro370, are located within the PEST domain. Three recombinant retroviruses expressing the mutated forms of Cx50 were generated (Fig. 2A): M5 contained mutations of all five residues, E328A, E331A, A348P, V362E, and P370A; M4 contained mutations on four residues, E328A, E331A, A348P, and P370A; and M3 contained deletion of the PEST motif (ΔPEST-Cx50) in conjunction with three additional mutations, E328A, E331A, A348P. By expressing wild-type and mutants of Cx50 in the primary lens culture using retroviral infection, we showed that only M4 had a similar stimulatory effect on lentoid formation as wild-type Cx50, whereas ΔPEST-Cx50, M3, and M4 mutants lack such effects when compared with wild-type Cx50 (Fig. 2B). These results were further verified by the expression level of AQP0 determined by immunofluorescence (Fig. 2C). M4 and
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**FIGURE 1. PEST of Cx50 CT is an important motif in lens fiber differentiation.** A, a schematic illustration of Cx50 lacking the PEST motif. B, the Cx50 and PEST motif-deleted mutant were co-expressed in primary lens cell culture by recombinant retroviral infection. The number of lentoids, which is an indication of lens epithelial-fiber differentiation, was quantified each day during the 8 days of culturing. C, at the 8th day, lens primary culture cells expressing exogenous Cx50, Cx50(–PEST) or vehicle, RCAS(A) control were fixed, immunolabeled with anti-AQP0 antibody, and followed by fluorescein-labeled secondary antibody. The immunofluorescence, AQP0-positive area was quantified. D, cell lysates from lens primary culture expressing E368A, E368D, S371A, and S371D were isolated and the expression level of AQP0 was immunoblotted using anti-AQP0 antibody (left panel) and quantified by densitometry (right panel). The data are presented as the mean ± S.E. n = 3. **, p < 0.01, in comparison with vehicle RCAS(A) control.

M5 have significantly different roles on lens cell differentiation and the only difference between these two mutants is the mutation on Val362. These data imply that Val362 is likely a critical residue in Cx50 CT that is functionally involved in lens fiber differentiation.

**Secondary Structural Prediction Suggests that Val362 Is Located in the Middle of an α-Helix Structure**—A homology model of structure for Cx50 CT was constructed using the atomic coordinates of cyclin A2 as a template in the SWISS-MODEL repository. This molecular modeling result suggested that the predicted structure of Cx50 CT contains four α-helical loops where all five highly conserved amino acid residues are located (Fig. 3A). Notably, only Val362 is located in the center of the 3rd helix, whereas all other conserved residues are located outside the helical structures. This finding implies that Val362 might be a critical residue in maintaining the helical structures at Cx50 CT.

As an aid to the homology modeling of Cx50 CT, other secondary prediction methods including SOPM were used to predict the Cx50 CT secondary structure primarily based on the composition of amino acids (15). These predictions suggested that about 60–70% of Cx50 CT contained α-helical structures, with four major helices (Fig. 3B, h in blue), which is consistent with the above molecular modeling analysis. Particularly, in all these predictions, Val362 is also positioned in the middle of the 3rd helical motif. In addition, Profile Hidden Markov Models were also employed to predict the possible secondary structure of Cx50 CT by scoring pairs of aligned secondary structure states in a way analogous to the classical amino acid substitution matrices. The predicted secondary structure of Cx50 CT by the HHpred program (16) shows good alignment between Cx50 CT and rabbit Cx43 CT with an E-value of 1.9E-12. The predicted helical motif containing Val362 is aligned within the 2nd helix of Cx43 CT (amino acids 342–348) (22) (Fig. 3C). The molecular modeling in conjunction with the structural prediction and alignment analysis suggested that Val362 is likely to be positioned within the putative 3rd helix of Cx50 CT.

Val362 Plays an Important Role in Maintaining Helical Structure and Lens Fiber Differentiation—To test if Val362 is critical in maintaining an α-helical structure and is crucial for the role of Cx50 in lens fiber differentiation, we generated single-site mutants by substituting Val362 with representative amino acid residues. Prior to functional assay, the mutation tool in the UCSF Chimera software package (36) was used to analyze the possible structural outcomes of these substitutions. The Rotamers tool allows amino acid side chain rotamers to be viewed and evaluated. “Best” rotamer is selected based on side chain torsion (ψ) and probability value, taken from the rotamer library, as well as in the context of the structural environment. The properties of amino acids we substituted are summarized in Table 3. Replacing Val362 with Arg, Lys, or Glu shows much lower possibilities (0.1–0.2) in maintaining the α-helix of Cx50 CT as compared with Leu, Thr, or Ile (0.6–0.8) (Fig. 4A). The changes of Val362 to a negatively charged residue, Glu, would disrupt the helical structure presumably due to an electrostatic interaction between two negatively charged residues. Interestingly, this modeling program predicts lower probability of α-helical content with either Arg or Lys substitution, even though both residues have relatively high α-helical propensity and would create a potentially favorable i, i + 4 electrostatic interaction (23). Conversely, changing Val362 to similar hydrophobic side chain residues, Val, Thr, or Ile, would not cause any structural alteration because two non-hydrogen substituents attached to their C-β carbon provides bulkiness near the protein backbone, thereby restricting the presence of these amino acids in the conformations adopted by the main chain. Interestingly, substituting Val362 with known α-helix breakers, Phe or Pro,
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FIGURE 2. Val362 is a likely residue involved in the role of Cx50 in promoting lens epithelial-fiber differentiation. A, based on the conserved residues between cyclin A and Cx50, three mutants of Cx50 were generated: M4, E328A, E331A, A348P, and P370A; but no V362E, ΔPEST-M3, ΔPEST-Cx50 with E328A, E331A, and A348P; and M5, all five highly conserved mutated residues, E328A, E331A, A348P, V362E, and P370A. B, recombinant retroviruses containing the wild-type and mutants were generated and used to infect lens primary cultures. Immunofluorescence (Fig. 4C) showed that these mutants are partially co-localized with endogenous Cx50. Substitution of Val362 with polar amino acids, Arg, Lys, and Glu significantly abolished the effect of Cx50 on increasing the number of lentoids (Fig. 4B), suggesting that all these mutants form functional gap junctions to a similar degree as wild-type Cx50, albeit their differences in promoting lens fiber differentiation. This result offers further support for the gap junction-independent role of Cx50 in lens epithelial-fiber differentiation as reported (11).

Mutations V362E, but Not V362T and V362P, Disrupted the α-Helical Structure Detected by CD Spectroscopy—We tested if Val362 is crucial for forming α-helical structures by examining the secondary structure contents of the peptides derived from putative 2nd and 3rd helices of Cx50CT using far UV CD spectroscopy. In aqueous solution, this peptide was largely unstructured. TFE induced the formation of α-helical structures with two major troughs at 208 and 222 nm observed in the CD spectra. The α-helical content increased to 34.4% in 30% TFE and to 45.7% in 50% TFE but remained the same in 70% TFE solution (Fig. 6A). TFE has been shown to induce and stabilize the intrinsic secondary structures in the peptides derived from connexins (25, 26), possibly by mimicking the plasma membrane-like hydrophobic environment. Sodium phosphate buffers with various pH were also tested in the CD assay and the data suggested that the wild-type peptide adopts the highest content of α-helical structures at low pH (pH 4.8) instead of pH 7.2 or 8.2 (Fig. 6B), presumably due to minimization of charge repulsion by the stretches of negatively charged glutamic acid residues found in the helical regions. In addition, the thermal stability of the peptide structures was determined at 0, 6, 25, and 37 °C and the peptide exhibited the highest α-helical content at low temperature (6 °C) (data not shown). The full CD spectrum of the wild-type and the other three mutant peptides, V362E, V362P, and V362T (Table 2), were obtained under the above optimized condition (50% TFE in 1 mM sodium phosphate buffer, pH 4.8, 6 °C) (Fig. 6C). Consistent with the molecular modeling studies, the peptides con-
Abolished the effect of Cx50 on differentiation. Structural modeling and analysis of CD spectra indicated that Val^{362} is crucial in maintaining an α-helical structure at Cx50 CT and disruption of this structure by altering the Val^{362} residue attenuated the role of Cx50 in differentiation. This study showed the importance of specific structural features of connexins in cellular function.

We have previously shown that only Cx50, but not Cx46 or Cx43, promotes chicken lens fiber differentiation (10). Further studies revealed that the C terminus of Cx50 is responsible for the role of Cx50 in promoting lens cell differentiation (11). However, the mechanism underlying this regulatory function was not clear. Here, we not only identified the involvement of specific motif and amino acid residues, but also demonstrated the importance of the integrity of a unique α-helical structure in the lens fiber differentiation process. This specific structural feature could be critical for binding of certain protein(s) important for differentiation.

The C termini of connexin molecules have been shown to be involved in various cellular functions. The C terminus of Cx43, an ubiquitously expressed connexin, has been shown to interact with several cellular proteins, such as ZO-1, p38 MAPK, calmodulin, tubulin, etc. (27). A previous study also showed that the C terminus of Cx50 directly interacts with ZO-1 although the function of this interaction is unclear (28). There is increasing evidence suggesting the role of connexins in regulating cell cycle regulators. Zhang et al. (29, 30) reported that the expression of Cx43 reduced the level of S phase kinase-associated protein 2 (Skp2), a factor important for cell cycle progression, and that this effect was independent of gap junction function. This is a likely mechanism for tumor suppressing functions of Cx43 on various tumor cell lines. We observed that culturing at days 4–5 is a critical time point for the stimulatory effect of Cx50 on lens epithelial-fiber differentiation. This implies that Cx50 is likely to be involved in the early stages of differentiation. One possibility is that Cx50 may be involved in the inhibition of fiber progenitor cell proliferation through cell cycle control, which in turn, leads to initiation and promotion of lens cell differentiation.

At the early stages of lens epithelial cell culture, both Cx43 and Cx50 are expressed. Due to technical reasons, we could not determine whether mutants associate with endogenous Cx50 to form heteromeric connexons, which may affect endogenous Cx50 function. However, this is unlikely because expression of exogenous wild-type Cx50 promotes differentiation. By co-immunostaining, we found that mutants had similar distribution patterns as wild-type and Cx50 are expressed. Due to technical reasons, we could not determine whether mutants associate with endogenous Cx50 function. This is a likely mechanism for tumor suppressing functions of Cx43 on various tumor cell lines. We observed that culturing at days 4–5 is a critical time point for the stimulatory effect of Cx50 on lens epithelial-fiber differentiation. This implies that Cx50 is likely to be involved in the early stages of differentiation. One possibility is that Cx50 may be involved in the inhibition of fiber progenitor cell proliferation through cell cycle control, which in turn, leads to initiation and promotion of lens cell differentiation.
its capability to promote lens epithelial-fiber differentiation, but has no effect on gap junctions. This is consistent with our previous studies that promotion of lens differentiation is independent of gap junctions using cataract-related mutants lacking gap junction function (11).

The three-dimensional structure of Cx43 CT has been reported using NMR spectroscopy, indicating two short helices (31). Based on Profile Hidden Markov Model alignment, we identified one short putative helical structure on Cx50 CT corresponding to the 2nd helical motif on Cx43 CT. Interestingly, this motif contains Val362. Our CD studies further confirm the importance of Val362 in sustaining a helical structure. Furthermore, we found that the integrity of this helical structure on Cx50 CT is directly correlated with the effect of Cx50 on lens fiber differentiation. Substitution of Val362 with charged amino acids reduces the helical content and consequently, abolishes the stimulatory effect of Cx50. One interesting observation here is that the substitution of Val362 with proline retains the helical structure although proline is typically known as a helix breaker. Due to the absence of the backbone amide hydrogen important for stabilizing an α-helix, proline is less favorable among 20 amino acids to form normal helical conformation. However, NMR studies verified the incorporation of a proline residue in the middle of an α-helix in the Oct-1 transcription factor. Modeling showed an altered H-bonding pattern, but the resulting helix was remarkably stable (32). Instead of valine at residue 362 in chicken, the proline residue is present in human and bovine ortholog forms of Cx50. Therefore, the helical structure is likely to be conserved across different animal species. Cellular function of proteins generally is fulfilled as a part of a complex with other protein molecules. The maintenance of certain structures of Cx50 could be critical to maintain the stability of the protein complex. Identifi-
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FIGURE 6. Val362 of Cx50 is critical in maintaining the helical motif assessed by CD spectroscopy. A, the far UV CD spectrum of the peptide containing Val362 in the presence of 0–70% TFE. Spectra were recorded at 6 °C in 1 mM sodium phosphate buffer (pH 4.8). B, the far UV circular dichroism spectra of the peptide in pH 4.8, 7, or 8.2, 1 mM sodium phosphate buffer in the presence of 50% TFE. All spectra presented in graphs were accumulated and averaged from 5 scans. The α-helical content of the peptides was calculated with the online server DICHROWEB (insets).

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REFERENCES
1. Tholozan, F. M., and Quinlan, R. A. (2007) Int. J. Biochem. Cell Biol. 39, 1754–1759
2. Mathias, R. T., Kistler, J., and Donaldson, P. (2007) J. Membr. Biol. 216, 1–16
3. Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996) Annu. Rev. Biochem. 65, 475–502
4. Gong, X., Cheng, C., and Xia, C. H. (2007) J. Membr. Biol. 218, 9–12
5. Kumar, N. M., and Gilula, N. B. (1996) Cell 84, 381–388
6. White, T. W., Goodenough, D. A., and Paul, D. L. (1998) J. Cell Biol. 143, 815–825
7. Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N. M., Horwitz, J., and Gilula, N. B. (1997) Cell 91, 833–843
8. Rong, P., Wang, X., Niesman, I., Wu, Y., Benedetti, L. E., Dunia, I., Levy, E., and Gong, X. (2002) Development 129, 167–174
9. Dunia, I., Cibert, C., Gong, X., Xia, C. H., Recouvreur, M., Levy, E., Kumar, N., Bloemendal, H., and Benedetti, E. L. (2006) Eur. J. Cell Biol. 85, 729–752
10. Gia, S., Yu, X. S., Yin, X., and Jiang, J. X. (2003) Invest. Ophthalmol. Vis. Sci. 44, 2103–2111
11. Banks, E. A., Yu, X. S., Shi, Q., and Jiang, J. X. (2007) J. Cell Sci. 120, 3602–3612
12. Jiang, I. X., White, T. W., Goodenough, D. A., and Paul, D. L. (1994) Mol. Biol. Cell 5, 363–373
13. Bruzzone, R., and Giaume, C. (eds) (2001) Methods in Molecular Biology, Vol. 154, pp. 159–174, Springer, Seacaucus, NJ
14. Jiang, I. X., and Goodenough, D. A. (1998) Invest. Ophthalmol. Vis. Sci. 39, 537–543
15. Geourjon, C., and Deléage, G. (1994) Protein Eng. 7, 157–164
16. Söding, J., Biegert, A., and Lupas, A. N. (2005) Nucleic Acids Res. 33, W244–W248
17. el-Fouly, M. H., Trosko, J. E., and Chang, C. C. (1987) Exp. Cell Res. 168, 422–430
18. Whitmore, L., and Wallace, B. A. (2008) Biopolymers 89, 392–400
19. Yin, X., Jedrzejewski, P. T., and Jiang, J. X. (2000) J. Biol. Chem. 275, 6850–6856
20. Yin, X., Yu, S., and Jiang, J. X. (2001) J. Biol. Chem. 276, 34567–34572
21. Zhou, L., Chen, T., and Church, R. L. (2002) Mol. Vis. 8, 143–148
22. Sorgen, P. L., Duffy, H. S., Spray, D. C., and Delmar, M. (2004) Biophys. J. 87, 574–581
23. Pace, C. N., and Scholtz, J. M. (1998) Biophys. J. 75, 422–427
24. Wallace, P., Signer, E., Paton, I. R., Burt, D., and Quinlan, R. (1998) Gene 211, 19–27
25. Zhou, Y., Yang, W., Lurtz, M. M., Ye, Y., Huang, Y., Lee, H. W., Chen, Y., Louis, C. F., and Yang, J. J. (2007) J. Biol. Chem. 282, 35005–35017
26. Fort, A. G., and Spray, D. C. (2009) J. Biol. Chem. 284, 6850–6856
27. Hirst-Jensen, B. J., Sahoo, P., Kieken, F., Delmar, M., and Sorgen, P. L. (2007) J. Biol. Chem. 282, 5801–5813
28. Cox, M., Dekker, N., Boelens, R., Verrijzer, C. P., van der Vliet, P. C., and Kaptein, R. (1993) Biochemistry 32, 6032–6040
29. Frishman, D., and Argos, P. (1996) Protein Eng. 9, 133–142
30. Yin, X., Jedrzejewski, P. T., and Jiang, J. X. (2000) J. Biol. Chem. 275, 392–400
31. Hirst-Jensen, B. J., Sahoo, P., Kieken, F., Delmar, M., and Sorgen, P. L. (2007) J. Biol. Chem. 282, 5801–5813
32. Cox, M., Dekker, N., Boelens, R., Verrijzer, C. P., van der Vliet, P. C., and Kaptein, R. (1993) Biochemistry 32, 6032–6040
33. Frishman, D., and Argos, P. (1996) Protein Eng. 9, 133–142
34. Guermeur, Y., Geourjon, C., Gallinari, P., and Deléage, G. (1999) Bioinformatics 15, 413–421
35. Deléage, G., Blanchet, C., and Geourjon, C. (1997) Biochimie 79, 681–686
36. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) J. Comput. Chem. 25, 1605–1612
37. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) Bioinformatics 22, 195–201
38. Notredame, C., and Higgins, D. (2000) J. Mol. Biol. 302, 205–217
39. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723