Characterization of the pH-dependent Interaction between the Gap Junction Protein Connexin43 Carboxyl Terminus and Cytoplasmic Loop Domains*

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A prevailing view regarding the regulation of connexin43 (Cx43) gap junction channels is that, upon intracellular acidification, the carboxyl-terminal domain (Cx43CT) moves toward the channel opening to interact with specific residues acting as a receptor site. Previous studies have demonstrated a direct, pH-dependent interaction between the Cx43CT and a Cx43 cytoplasmic loop (Cx43CL) peptide. This interaction was dependent on α-helical formation for the peptide in response to acidification; more recent studies have shown that acidification also induces Cx43CT dimerization. Whether Cx43CT dimerization is an important structural component in Cx43 regulation remains to be determined. Here we used an assortment of complimentary biophysical techniques to characterize the binding of Cx43CT or its mutants to itself and/or to a more native-like Cx43CL construct (Cx43CL 100–155 residues 100–155). Our studies expand the observation that specific Cx43CT domains are important for dimerization. We further show that properties of the Cx43CL 100–155 are different from those of the Cx43CL peptide; solvent acidification leads to Cx43CL 100–155 oligomerization and a change in the stoichiometry and binding affinity for the Cx43CT. Homo-Cx43CT and Cx43CL 100–155 oligomerization as well as the Cx43CT/Cx43CL 100–155 interaction can occur under in vivo conditions; moreover, we show that Cx43CL 100–155 strongly affects resonance peaks corresponding to Cx43CT residues Arg-376—Asp-379 and Asn-343—Lys-346. Overall, our data indicate that many of the sites involved in Cx43CT dimerization are also involved in the Cx43CT/Cx43CL interaction; we further propose that chemically induced Cx43CT and Cx43CL oligomerization is important for the interaction between these cytoplasmic domains, which leads to chemically induced gating of Cx43 channels.

Gap junctions are integral membrane proteins that enable direct cytoplasmic exchange of ions and low molecular weight metabolites between adjacent cells. They provide a pathway for the propagation and/or amplification of signal transduction cascades triggered by cytokines, growth factors, and other cell signaling molecules involved in growth regulation and development. Mutations in the gap junction proteins have been associated with hereditary non-syndromic deafness, Charcot-Marie-Tooth disease, ocudolentodigital dysplasia, and cataracts, among other congenital human diseases (1). Gap junctions are formed by the apposition of connexons from adjacent cells, where each connexon is formed by six connexin proteins. Connexins are tetraspan transmembrane domain proteins with intracellular amino and carboxyl termini. There are 21 different connexin genes in the human genome. Although there is significant sequence homology among connexins, the major divergence in primary structures occurs in the cytoplasmic domains. The 43-kDa protein connexin43 (Cx43) is the most abundant gap junction protein in various tissues (e.g. heart and brain) and is the focus of the present study.

The importance of Cx43 gap junctions in the cardiovascular system is well established. In the heart they allow for the propagation of the action potential and the maintenance of a regular rhythm. If Cx43 channels close or are not present, normal propagation is disrupted, and lethal arrhythmias can ensue (2–4). The presence and proper regulation of Cx43 channels are also essential for normal cardiac embryogenesis (5). Cx43 channels can be regulated in a variety of ways; for example, they are regulated by kinases (6). Cx43 channels can also be regulated by other molecules and physiological conditions, such as variations in Ca 2⁺, pH, intercellular voltage, cyclic nucleotides, neurotransmitters, and hormones (7–11).

Intracellular acidification in particular leads to channel closure of gap junctions in all native tissues and exogenous expression systems tested (12–14). The study of pH-dependent regulation of gap junctions becomes even more relevant given that intracellular acidification is a major consequence of tissue ischemia (15). Experimental models of acute ischemia show that electrical uncoupling develops within 15 min after the onset of the ischemic event (16). Uncoupling occurs before any detectable changes in the integrity of the gap junction plaque (17), suggesting that gap junction channels close while still in the membrane and before being internalized (18). Acidifica-

2 The abbreviations used are: Cx43, connexin43; Cx43CT, connexin43 carboxyl terminus; Cx43CL, connexin43 cytoplasmic loop; B5 1, bis(sulfosuccinimidyl) suberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride; HSQC, heteronuclear single-quantum correlation; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; ZO-1, zona ocludens-1; MTS, 1-oxyl-2,2,5,5-tetramethyl-pyrroline-3- methylmethanethiosulfonate.

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Cx43CT/Cx43CL Interaction

Prenatal uncoupling also has an impact on the preservation of tissue surrounding the ischemic area in the heart and may be a key substrate for life-threatening arrhythmias during myocardial infarction (19).

pH-induced closure of Cx43 channels involves interaction of the Cx43 carboxyl-terminal domain (Cx43CT) with a separate "receptor" domain. Based on the range of pH values corresponding to gating, histidine residues, particularly those located within the Cx43 cytoplasmic loop (Cx43CL), were proposed to be the candidates for the pH sensor (8). Work performed in Xenopus oocytes showed that a Cx43CT truncation prevented acidification-induced uncoupling (20). Co-expression of the Cx43CT as a separate fragment partially recovered this function, demonstrating the possible involvement of intramolecular interactions in pH gating (20). These data led to the notion that pH gating results from a "particle-receptor" interaction, modeled after the "ball-and-chain" mechanism of voltage-dependent inactivation (21). In this case, the Cx43CT would act as the gating particle that, under appropriate conditions, would bind to a receptor affiliated with the pore. Further studies showed a direct, pH-dependent interaction between the Cx43CT and a peptide corresponding to the second half of the Cx43CL (22). This interaction was dependent on the formation of an α-helical structure for the Cx43CL peptide (two histidines present) in response to acidification. We then proposed that the Cx43CL corresponds to the receptor for the Cx43CT (22). Recent patch clamp studies have supported this hypothesis (23).

Although a direct intramolecular interaction between the Cx43CT and Cx43CL was observed, we hypothesize that this interaction represents only part of the structural foundation for the particle-receptor hypothesis of pH gating. Nuclear magnetic resonance (NMR) transdiffusion experiments indicated reduced mobility for the Cx43CT fragment at low pH, which could be explained by the formation of a higher molecular weight species (22). Further studies applying a variety of biophysical methods demonstrated that the Cx43CT dimerized in a pH-dependent manner (24). Hence, dimerization of the Cx43CT may be one of the structural changes involved in the pH regulation of Cx43. In particular, we proposed that dimerization would increase the Cx43CT/Cx43CL binding affinity to bring the channel to a closed state and that the change in oligomeric state may play a role in modulating the molecular partners that associate with Cx43 under a given condition, thus acting as a switch for modifications in channel function.

In this study we have used chemical cross-linking, sedimentation equilibrium, surface plasmon resonance, yeast two-hybrid, and NMR to characterize molecular events associated with chemical regulation of Cx43. Using both Cx43CT and Cx43CL wild-type constructs and a collection of Cx43CT mutants, we demonstrate that specific domains of the Cx43CT are important for pH-dependent dimerization. We further show that the properties of a more native-like Cx43CL construct are different from those of the previously characterized Cx43CL peptide (22); in particular, our results demonstrate that solvent acidification leads to Cx43CL wild-type oligomerization, which in turn affects the stoichiometry and binding affinity for the Cx43CT. In addition, we show that homo-Cx43CT and Cx43CL oligomerization as well as the Cx43CT/Cx43CL interaction can occur within the cellular environment. Finally, our NMR studies led us to identify the Cx43CT residues that are affected by the interaction with the Cx43CL. We hypothesize that the pH-dependent structural modification of both the Cx43CT and Cx43CL domains is necessary for their association, which eventually leads to the closure of Cx43 gap junctions in response to low pH.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Glutathione S-Transferase-Cx43CT—The Cx43CT wild-type, Cx43CT mutants, 15N-labeled Cx43CT, and Cx43CL100–155 polypeptides were expressed and purified as described previously (22). All polypeptides were confirmed for purity and analyzed for degradation by SDS-PAGE.

Chemical Cross-linking—Cross-linking of Cx43CT mutants, Cx43CL100–155, and Cx43CT mutants-Cx43CL100–155 was carried out for 1 h at room temperature using either 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) or 1 mM bis(sulfosuccinimidyl) suberate (BS3). The reactions occurred in phosphate-buffered saline (PBS) at pH 5.8 and 7.5 and were quenched by the addition of ethanolamine-HCl to a final concentration of 100 mM. Complete quenching was achieved by leaving the reactions standing for 10 min at room temperature followed by heating in SDS sample buffer. The products of the reaction were then separated on 20% SDS-PAGE gels and bands visualized by Coomassie Blue staining.

Using Quantity One software (Bio-Rad), the areas containing monomer and dimer Cx43CT wild type and mutants for pH 5.8 were highlighted while keeping the area constant. The volumes (mm² × intensity), after adjusting for background, were used to calculate the percentage of dimer with respect to monomer for each Cx43CT mutant. This method accounts for minor variations in the amount of protein loaded on the SDS-PAGE gel and allowed us to quantitatively compare the dimerization differences in the Cx43CT mutants.

Analytical Centrifugation—Sedimentation equilibrium experiments were performed using a Beckman ProteomeLab XL-I analytical ultracentrifuge and an AN-60Ti rotor available at the University of Nebraska Medical Center Analytical Ultracentrifuge Shared Resource Facility. The Cx43CT mutants were analyzed at 25 °C in PBS buffer (pH 5.8 and 7.5). For each condition data were collected at three concentrations (0.3, 0.5, and 0.9) and two rotor speeds (18,000 and 26,000 rpm). Absorbance scans at 280 nm were taken after 14, 22, and 24 h at each speed; it was assumed that equilibrium was reached if the scans were unchanged. Data analysis was performed using the Beckman XL-A/XL-I software package within Microcal, ORIGIN Version 4. Each analysis consisted of six absorbance scans taken at three different nominal concentrations and at each of the two rotor speeds. Buffer densities were determined using a Mettler DE40 density meter operated at the experimental temperature, and data were analyzed with the program Sednterp v1.03. Partial specific volume was calculated in Sednterp from the amino acid composition.

Surface Plasmon Resonance (SPR)—All SPR experiments were performed using a Biacore 3000 at the University of
Nebraska Medical Center Molecular Interaction Facility. Cx43CT wild-type and mutants or Cx43CL in 1 mM maleate (pH 7.0) were immobilized covalently on the sensor chip CM-5 with EDC and N-hydroxysuccinimide according to the manufacturer’s specifications (Biacore AB, Sweden). In each flow cell 2 nmoI of protein were loaded onto the surface, resulting in ~2000 ‘response units’ of protein captured. The amount of protein on each flow cell was calculated to be 0.22 pmol/mm² ± 1%. The sensor chip, including an activated flow cell for reference, was capped with an addition of 35 µl of 1 M ethanolamine.

All analysis was performed at a flow rate of 20 µl/min. Before the loading of analytes, the chip was equilibrated with PBS containing 0.005% surfactant P-20 (PBS-P) (pH 5.8 or 7.5). Each analyte at various concentrations in the same buffer was injected in duplicate at random over the immobilized ligand. After injection of the analyte, PBS-P was reintroduced with a 300-s lag time to start dissociation. The chip was regenerated to base line by injection of 5 µl of 100 mM HCl or 0.1 M NaHCO3 (pH 8.6).

Response curves were generated by subtraction of the background signal generated simultaneously by the reference flow cell. In both phases (association and dissociation), the first 5–8 s of recording were not included in the fit as to avoid artifacts resulting from peptide distribution within the flow cell. Association and dissociation rate constants (ka and kd) for the Cx43CL/Cx43CL and Cx43CT/Cx43CT interactions at pH 5.8 were calculated simultaneously from 6 curves of different concentrations of analyte by using the 1:1 Langmuir binding model in the BIAevaluation 4.1 software (Biacore). The best fit was determined from the amplitude and order of the residual data. For all best fits residual data distributed randomly and departed from the model by less than 1% (in relative response units amplitude). Calculated fits were discarded if χ-squared values were higher than 2 units/curve. The software uses numerical integration algorithms to generate ka and kd. The affinity constant (Kd) was calculated from the ka and kd. The affinity constant (Kd) was calculated from the ka and kd.

Yeast Two-hybrid Assay—The Saccharomyces cerevisiae strain AH109 (BD Biosciences Clontech) was maintained on yeast extract, peptone, and dextrose agar plates. Co-transformation was done by the lithium acetate procedure as described in the instructions for the MATCHMAKER two-hybrid kit (BD Biosciences Clontech). For colony growth assays, AH109 cotransformants were streaked on plates lacking leucine and tryptophan and allowed to grow at 30 °C usually for 3 days or until colonies were large enough for further assays. An average of three to four colonies was then chosen and suspended in water, equilibrated to the same optical density 600 nm, and replated on plates lacking leucine and tryptophan (+HIS) as well as plates also lacking histidine (−HIS).

NMR—NMR data were acquired at 7 °C using a 600-MHz Varian INOVA NMR spectrometer fitted with a cryo-probe at the University of Nebraska Medical Center NMR Core Facility. Gradient-enhanced two-dimensional 1H/15N HSQC experiments (25) were used to observe all backbone amide resonances in 15N-labeled Cx43CT. Data were acquired with 1024 complex points in the direct dimension and 128 complex points in the indirect dimension. Sweep widths were 10,000 Hz in the proton dimension and 2500 Hz in the nitrogen dimension. NMR spectra were processed using NMRPipe (26) and analyzed using NMRView (27).

RESULTS

Preparation of Recombinant Cx43CT Mutants—Previous studies from our laboratory characterizing the pH-dependent regulation of Cx43 gap junction channels revealed pH-dependent Cx43CL α-helical formation (22), Cx43CT dimerization (24), and a Cx43CT/Cx43CL interaction (22). To further characterize these events, we generated a series of recombinant Cx43CT deletion mutants (Fig. 1, top). The mutants were designed to model deletions of the dimerization domains (Cx43CT Δ281–295, Δ299–304, Δ314–327, and Δ340–348) (24), the two helical domains (Cx43CT Δ314–327, Δ340–348, and Δ314–327,340–348) (28), the ZO-1 PDZ-2 binding domain (Cx43CT Δ364–382) (28), a region of currently unknown importance (Cx43CT Δ348–363), a carboxyl-terminal truncation mutant (Cx43CT Δ315–382), and a series of amino-terminal truncation mutants (Cx43CT Δ255–279, Δ255–295, and Δ255–313). Each of the Cx43CT mutant constructs were purified to homogeneity (Fig. 1, bottom). For clarity, the numbering of amino acids in full-length Cx43 was retained for the recombinant Cx43CT proteins.

Chemical Cross-linking of Cx43CT Mutants—Although previous NMR studies revealed Cx43CT residues involved in dimerization (24), we were unable to identify sufficient intermolecular energy transfers by the nuclear Overhauser effect to generate models of the dimer structure. This may be due to spectral overlap as well as the dimer interaction being weak and transient. Therefore, to identify the minimal region(s) necessary for dimer formation and possible additional dimerization domains, recombinant Cx43CT mutants were exposed to the irreversible cross-linking agent EDC (Fig. 2A). EDC is a zero-length cross-linking agent used to couple carboxyl groups to primary amines. The reaction was conducted at two different pH values (5.8 and 7.5). To assure the accuracy of the trends in intensity changes and quantitate the cross-linking data, cross-linking for the wild type and each of the mutants was repeated in triplicate (data not shown), and densitometry was performed to determine the fraction of total protein found in the dimer form. The percent dimerization indicated in Fig. 2A is the average of the three cross-linking replicates.

Cross-linking with the expected mobility for dimers was observed for each of the Cx43CT deletions at pH 5.8 except for the Cx43CT Δ315–382 mutant. However, when compared with wild type, the intensity of the dimer band significantly decreased for Cx43CT mutants Δ314–327, Δ314–327/Δ340–348, and Δ364–382 (~69% decrease), moderately decreased for Cx43CT mutants Δ281–295 and Δ299–304 (~28% decrease), and increased for the amino-terminal Cx43CT truncation mutants Δ255–280, Δ255–295, and Δ255–313. At pH 7.5 some dimeric species were observed; however, oligomerization was much more prevalent at the low pH, consistent with our previous studies, suggesting a pH dependence to the dimerization process (24). The involvement of Cx43CT residues 364–382 in dimerization, which was not previously identified by NMR (unlike Cx43CT residues 281–295, 299–304, and 314–327...
was confirmed by SPR and EDC cross-linking of Cx43CT wild type to a peptide containing Cx43CT residues 364–382 (data not shown). Together, these results suggest that, rather than being localized to a single Cx43CT region, dimerization is a more global phenomenon that depends on the overall integrity of the carboxyl terminus domain.

To confirm the results obtained in the presence of EDC (Fig. 2A), two Cx43CT mutants (Δ314–327/340–348 and Δ255–295) were assessed for cross-linking using a different cross-linking agent (BS3). This chemical was chosen over EDC because of its different optimal pH range (7.0–9.0 versus 4.5–7.5), spacer arm length (11.4 Å versus 0 Å), and reactive group (only primary amines versus primary amines and carboxyl groups). As shown in Fig. 2B, there was an increase in band density of the Δ255–295 dimer and a decrease for Δ314–327/340–348 when compared with wild type. Interestingly, trimeric species were observed for the wild type and not the Cx43CT mutant Δ255–295. These results indicate that the BS3 cross-linking pattern for these mutants was consistent with that of the EDC cross-linking. Because of their distinctive effects, these mutants were used for further characterization of Cx43CT dimer formation.

To characterize the binding affinity of the Cx43CT/Cx43CT interaction, SPR was performed (Fig. 2C). In this method an interaction between the ligand bound to the chip and analyte flowing over the chip results in an increase of mass. The signal is measured in arbitrary response units. Cx43CT was covalently bound to a carboxymethyl dextran matrix on the surface of a sensor chip and exposed to different concentrations of Cx43CT at pH 5.8. In agreement with the cross-linking data, Cx43CT interacted with itself at pH 5.8. The \( K_D \) value of the Cx43CT/Cx43CT interaction at pH 5.8, estimated by using the 1:1 Langmuir binding model, was 0.21 \( \mu \)M.

**Molecular Mass Determination for Cx43CT Mutants**—A more quantitative analysis of the effect of specific mutations on Cx43CT dimerization was obtained by sedimentation analysis. This method also allowed us to more accurately determine the stoichiometry of oligomerization at different pH values. Plots of optical density at equilibrium as a function of radius for Cx43CT mutants Δ314–327/340–348 and Δ255–295 are presented in Fig. 3A. Each plot was best fitted by a function derived from a self-association model to determine the fraction of protein in specific oligomeric states (Beckman XL-A/XL-I software package). A monomer-dimer-trimer model showed excellent convergence, as demonstrated by the minimal deviation seen in the residuals (top plot on each panel) and a weighted variance approaching unity (data not shown). In contrast, fits of data to single component (i.e. non-oligomeric), monomer-dimer, and monomer-dimer-tetramer models had weighted variance values significantly greater than one. The results for the monomer-dimer-trimer model were used to calculate the fraction of total protein that existed in the dimer conformation at a concentration of 70 \( \mu \)M (the same concentration as used in the cross-linking experiment in Fig. 2) at pH 5.8 and 7.5 (Fig. 3B). As compared with Cx43CT wild-type (86% dimer, pH 5.8, and 12% dimer, pH 7.5 (24)), the Cx43CT Δ314–327/340–348 mutation had an overall decrease in dimer formation, and the Cx43CT Δ255–295 mutation had an increase in dimer formation at both pH values. These results were qualitatively consistent with those obtained by cross-linking (see Fig. 2), although quantitative differences were also apparent (see “Discussion”).
Characterizing Cx43CL Oligomerization—Previous studies to determine the receptor domain for the Cx43CT used a peptide corresponding to the second half of the Cx43CL (dubbed L2; Cx43CL_{119–144}, residues 119–144 (22)). Although the Cx43CL_{119–144} peptide allowed us to ascertain that the two domains interact under acidic conditions, a better system was needed to fully characterize the structural basis of the particle-receptor interaction (see “Discussion”). Therefore, we cloned the entire CL of Cx43 (residues 100–155; 31 residues longer than the Cx43CL peptide) into the expression vector pGEX-6P-2 and purified this construct by standard glutathione S-transferase protocols. We initially characterized this more “native-like” Cx43CL using chemical cross-linking (Fig. 4A) and SPR (Fig. 4B) to determine whether a similar mechanism of pH-dependent self-oligomerization exists as has been determined for the Cx43CT (24). The Cx43CL_{100–155} was combined with EDC and BS{sup 3} at pH 5.8 and 7.5 (Fig. 4A). An ~15-kDa band was apparent in the cross-linking lanes at low pH and was (very) faintly present in the EDC lane at pH 7.5. The cross-linked bands had the expected mobility for dimers of Cx43CL_{100–155}. Also present in the EDC lane at pH 5.8 were (very) faint cross-linking bands that corresponded to trimers and tetramers of Cx43CL_{100–155}. The Cx43CL_{100–155} has a molecular mass of 7154 Da, as determined by mass spectroscopy; however, the monomeric species ran at a higher molecular mass in the 20% SDS-PAGE, possibly due to the high number of positive charges.

To confirm pH-dependent Cx43CL_{100–155} oligomerization and characterize the binding affinity of the interaction, SPR was performed (Fig. 4B). Cx43CL_{100–155} was covalently bound to a carboxymethyl dextran matrix on the surface of a sensor chip and exposed to different concentrations of Cx43CL_{100–155} at pH 5.8 and 7.5. In agreement with the cross-linking data, Cx43CL_{100–155} interacted with itself at pH 5.8, whereas a significant reduction in the amplitude of the response was evident by increasing the solvent pH to 7.5. These data suggest that low pH increases the binding affinity of the Cx43CL_{100–155} for itself. The \( K_D \) value of the Cx43CL_{100–155}-Cx43CL_{100–155} interaction at pH 5.8, estimated by using the 1:1 Langmuir binding model, was 0.38 \( \mu \)M. The amplitude of the binding curve at pH 7.5 was too small to be properly fit; therefore, \( K_D \) values for the binding at pH 7.5 could not be calculated under these conditions. Altogether, the results demonstrate selective, pH-dependent homomeric binding of the Cx43 cytoplasmic loop.

Characterizing the Cx43CT/Cx43CL Interaction—The ability of the Cx43CL_{100–155} construct to interact with Cx43CT was assessed by cross-linking. Binding was compared with that.
Either the purified Cx43CL100–155 polypeptide or the Cx43CL119–144 peptide were mixed with Cx43CT wild type at a 3:1 molar ratio and combined with the cross-linking agent EDC. As shown in Fig. 5, bands corresponding to a 1:1 Cx43CT to Cx43CL binding were observed (black arrow) for the interaction of Cx43CT with both Cx43CL100–155 (panel A) and the Cx43CL119–144 peptide (panel B).

FIGURE 3. Distribution of Cx43CT mutants at sedimentation equilibrium. A, the concentrations of Cx43CT mutants Δ314–327/340–348 and Δ255–295 in PBS buffer (pH 5.8 and 7.5) at equilibrium (26,000 rpm) are shown as a function of radius. The solid lines are the theoretical curves. B, extent of Cx43CT mutant Δ314–327/340–348 and Δ255–295 oligomerization as a function of the pH of the solvent.

FIGURE 4. Characterization of pH-dependent Cx43CL self-association by chemical cross-linking and SPR. A, Cx43CL100–155 was present at a concentration of 1.0 μg/μl in PBS buffer (pH indicated above each panel) in the presence (+) or absence (-) of EDC or BS3. Equal amounts of protein (10 μg) were electrophoresed on a 20% SDS-PAGE and stained with Coomassie Blue. The molecular mass standards for the 10-, 15-, 20-, and 25-kDa bands have been labeled. B, sensograms obtained after adding 2.2 and 11 μM Cx43CL100–155 at pH 5.8 and 7.5 to a cuvette where Cx43CL100–155 had been immobilized.

of the Cx43CL119–144 peptide (22). Either the purified Cx43CL100–155 polypeptide or the Cx43CL119–144 peptide were mixed with Cx43CT wild type at a 3:1 molar ratio and combined with the cross-linking agent EDC. As shown in Fig. 5, bands corresponding to a 1:1 Cx43CT to Cx43CL binding were observed (black arrow) for the interaction of Cx43CT with both Cx43CL100–155 (panel A) and the Cx43CL119–144 peptide (panel B). However, unlike the case of the Cx43CL119–144 peptide, a band correlating to 2 Cx43CT cross-linked
to 1 Cx43CL_{100–155} was also present (gray arrow). We speculate that the additional Cx43CL residues are important for a stronger binding affinity between the Cx43CT and Cx43CL domains.

SPR was used to confirm the association and calculate the binding affinity for the Cx43CT/Cx43CL_{100–155} interaction. Cx43CT wild type (Fig. 5C) and Cx43CL_{100–155} (Fig. 5D) were covalently bound to a carboxymethyl dextran matrix on the surface of a sensor chip and exposed to different concentrations of Cx43CL_{100–155} and Cx43CT, respectively, at pH 5.8. In agreement with the cross-linking data, the Cx43CT interacted with the Cx43CL_{100–155}. The interaction between Cx43CT and Cx43CL_{100–155} however, represented a more complicated system when attempting to determine the binding affinity (K_D). Global fitting of the curves (independent of orientation, Figs. 5, C and D) with the methods provided in the BIAevaluation 4.1 software (Langmuir 1:1, Bivalent-Analyte, Hetero-ligand, Conformational Change, and Hetero-Analyte methods) could not accurately model the interaction to determine the K_D (χ^2 was >5000 units/curve for each model). This suggests that, as predicted by the cross-linking results, the interaction between Cx43CT and Cx43CL_{100–155} is not properly described by equations based on first-rate order kinetics.

Next, the yeast two-hybrid method was used to identify if the Cx43CT and Cx43CL_{100–155} domains physically associate in yeast cytoplasm (Fig. 6). The Cx43CT and Cx43CL_{100–155} domains were co-expressed in AH109 yeast cells as a GAL4 DNA binding domain fusion protein (Fig. 6, bd) and as a GAL4 transactivation domain fusion protein (ad), respectively. In separate experiments, the reverse combination was used (Cx43CT as the transactivator and Cx43CL_{100–155} as the GAL4 DNA-binding protein). In either configuration, the Cx43CT interacted with the Cx43CL_{100–155}. Additionally, the yeast two-hybrid method confirmed both homo-oligomerization between Cx43CT domains and Cx43CL_{100–155} domains.

Finally, we used chemical cross-linking and SPR to test whether Cx43CT binds in a pH-dependent manner to the Cx43CL_{100–155}. The Cx43CL_{100–155} was mixed with wild-type Cx43CT at a 2:1 molar ratio and combined with the cross-linking agent EDC at pH 5.8 and 7.5. As shown in Fig. 7A, bands corresponding to a 1:1 Cx43CT to Cx43CL (black arrow) and Cx43CT to Cx43CT (gray arrow) binding were observed at both pH values; however, the intensity of these bands significantly decreased at pH 7.5.
SPR was used to confirm the pH-dependent association between the Cx43CT and Cx43CL100–155. Cx43CL100–155 was covalently bound to a carboxymethyl dextran matrix on the surface of a sensor chip and exposed to different concentrations of Cx43CT at pH 5.8 and 7.5 (Fig. 7B). In agreement with the cross-linking data, the Cx43CT interacted with the Cx43CL100–155 at pH 5.8 and 7.5; however, a significant reduction in the amplitude of the response was evident by increasing the solvent pH to 7.5. Although the $K_d$ cannot be determined for the Cx43CT/Cx43CL100–155 interaction (as described above), the amplitude of binding suggests a higher affinity interaction under acidic conditions.

**Residues Involved in the Cx43CT/Cx43CL Interaction**—To determine the location of the Cx43CT/Cx43CL interaction, we employed three complimentary biophysical techniques: chemical cross-linking, NMR, and SPR. Initially, the Cx43CL100–155 polypeptide was used to cross-link with each of the Cx43CT mutations at a 1:1 molar ratio (Fig. 8A). Although the density of the band corresponding to the cross-linked species varied, most Cx43 mutants were able to cross-link with the Cx43CL100–155 (bands labeled by CT-CL, 1:1 molar ratio, in bold). The exceptions, as compared with Cx43CT wild type, were mutant Cx43CT Δ314–327/340–348, which showed a significant decrease, and the Cx43CT Δ364–382 and Δ315–382 mutants, which showed no cross-linking to the Cx43CL100–155. A common feature for each of these three mutants is a correlation between decreased Cx43CL cross-linking (Fig. 8A) and decreased Cx43CT dimerization (Fig. 2A).

An immunoblot experiment using an anti-Cx43CT antibody (residues 360–382) confirmed the location of the Cx43CT/Cx43CL100–155 cross-linked band (Fig. 8B). An immunoblot for the cross-linking of Cx43CT wild type without Cx43CL100–155 was also included to better establish the location of the Cx43CT/Cx43CL100–155 protein (1:1 molar ratio, in bold).
within the blot. The data show an ~20-kDa band that was only present when cross-linking was performed in the presence of Cx43CL100–155. Additionally, a band of this size was not observed when the Cx43CL100–155 (Fig. 2A, wild-type lane) or EDC (Fig. 8A, Control lane) was not present as well as when the Cx43CL100–155 was cross-linked alone (Fig. 4A).

NMR was used to determine whether the Cx43CT regions 314–327, 340–348, and/or 364–382 were directly involved in the binding of Cx43CT to Cx43CL100–155. 15N-Labeled Cx43CT was diluted in PBS buffer (pH 5.8) containing spin-labeled attached 14N-labeled Cx43CL100–155 to a final 1:2 molar ratio, and 15N-labeled HSQC spectra were acquired (Fig. 9A). The 15N-labeled HSQC is a two-dimensional NMR experiment in which each amino acid (except proline) gives one signal that corresponds to the N-H amide group. These chemical shifts are sensitive to their environment and can be modified by small changes in structure and/or dynamics.

The spin label agent MTSL (1-oxyl-2,2,5,5-tetramethyl-p-pyrroline-3-methylmethanethiosulfonate) was covalently attached to an amino-terminal Cys residue that was inserted before Cx43CL residue Met-100. The Cx43CT is predominantly a disordered protein (28), and as such, many important protein contacts may take place at distances far exceeding the useful range of conventional nuclear Overhauser effects or outside the detection level of chemical shift changes that would be observed in a 15N-labeled HSQC (29). A spin-label experiment detects the interaction between the unpaired electron from the MTSL and the nucleus from the Cx43CT. Therefore, transient contacts up to 20 Å in distance can be mapped (30).

The Cx43CT resonance contours acquired in the absence of the MTSL-Cx43CL100–155 are depicted in black in Fig. 9A. Contours obtained in the presence of the MTSL-Cx43CL100–155 are labeled red. MTSL alone had no affect on the Cx43CT resonance peaks (data not shown). In previous studies we have assigned the specific resonance peaks that correspond to each amino acid in the Cx43CT sequence (31). Accordingly, shifts in the resonance assignments directly reveal the identity of the amino acids whose position in space is modified by the presence of Cx43CL100–155. As shown in Fig. 9A, the addition of Cx43CL100–155 strongly affected the resonance peaks of Cx43CT residues Arg-376, Asp-378, and Asp-379 and residues Asn-343, Ala-344, Lys-345, and Lys-346. In addition, there were minor shift between amino acids Asn-312—Ala-332, Phe-335—Asp-340, Val-347—Ala-349, Ala-357—Gln-361, Ser-
364—Arg-374, and Leu-380—leu-382. It is important to note that residues Pro-338, Pro-363, Pro-375, and Pro-377 do not provide an identifiable resonance peak because they do not contain an amide bond; yet, these residues may also be affected by the Cx43CT-Cx43CL interaction.

The same experiment was performed using an MTSL-labeled Cx43CL119–144 peptide (Fig. 9B). The Cx43CT residues affected by this peptide were also affected by Cx43CL100–155. The major differences were that (a) the Cx43CT resonance shifts caused by Cx43CL119–144 were limited to only those residues that were “strongly affected” by Cx43CL100–155, and (b) the shifts were smaller, indicating a lower affinity interaction. Similar to the cross-linking, these data suggest that the additional Cx43CL residues are important for a stronger binding affinity between these cytoplasmic domains.

SPR was used to determine whether the Cx43CT regions affected by the interaction with Cx43CL100–155 (as per the NMR experiments) were necessary for Cx43CT/Cx43CL100–155 binding. Cx43CT mutants Δ340–348 and Δ364–382 were covalently bound to a carboxymethyl dextran matrix on the surface of a sensor chip and exposed to different concentration of Cx43CL100–155 at pH 5.8 (Fig. 10). Compared with the response in the presence of the wild-type Cx43CT protein, the amplitude of the resonance signal elicited by Cx43CL100–155 was significantly decreased when the chip was loaded with mutant Cx43CT Δ364–382 or with mutant Cx43CT Δ340–348. These results suggest the Cx43CL100–155 has an affinity for multiple Cx43CT regions, with the dominant site located within the 364–382 region.

DISCUSSION

This investigation was undertaken to provide insight into the structural and functional role of the Cx43 intracellular domains in the chemical regulation of Cx43 gap junction channels. The results support the notion that oligomerization plays a role in the interdomain interactions necessary for Cx43 regulation and identified the structural determinants necessary for these interactions. The data further support the particle-receptor model for Cx43 chemical gating and emphasizes the importance of structural modifications for both the Cx43CT and Cx43CL domains in pH gating.

Cx43CT Dimerization Domains—Our studies to determine which Cx43CT region(s) is critical for dimerization were motivated by our previous NMR experiments, which identified four specific dimerization domains: Met-281—Asn-295, Arg-299—Gln-304, Ser-314—Ile-327, and Gln-342—Ala-348 (24). Although no one region was identified as essential for dimerization, the cross-linking and sedimentation equilibrium data confirmed that Cx43CT residues Ser-314—Ile-327 were involved in the dimerization process. Interestingly, region Ser-314—Ile-327 also corresponds to an area of the primary sequence where high-order structure has been identified (24). We speculate that dimerization may involve formation of a coiled-coil structure between the two subunits. Moreover, our mutagenesis analysis suggests a role for region Ser-314—Ile-327 in dimerization. Structural involvement of this region was not detected in our NMR experiments (24), likely because of spectral overlap caused by three SSR repeats in this short sequence. These data suggest that contact between two Cx43CT domains can occur throughout most of the length of the Cx43CT. Our analysis also indicates that deletion Δ315–382 was the only mutation for which dimerization was completely absent, whereas the presence of Cx43CT residues Met-281—Asn-295 and Arg-299—Gln-304,
which moderately decreased Cx43CT dimerization when deleted, was not sufficient to induce dimerization. This may suggest that the Ser-314—Ile-327 and Ser-364—Ile-382 regions are critical for the nucleation of dimerization, whereas regions Met-281—Asn-295 and Arg-299—Gln-304 may modulate the affinity of one subunit for the other. It is worth noting that failure to detect dimerization may be a technical limitation of using soluble protein fragments from a membrane protein.

An important question that arises from studying the structure of a soluble domain of a membrane protein is whether it has the same characteristics as when attached to the membrane. Numerous studies have demonstrated that the Cx43CT domain retains at least some of its functional properties (e.g., phosphorylation, binding molecular partners, and the rescue of pH sensitivity of truncated Cx43 channels (6, 20, 32)), yet one likely difference is that the amino terminus of the Cx43CT will become ordered or structured (i.e., less flexible) when attached to the fourth transmembrane domain. The electron crystallographic structure of a CT-truncated Cx43 mutant (stop at Thr-263) suggested that region Ser-255—Thr-263 adopted an α-helical structure similar to the fourth transmembrane domain (33); yet, this structure is not identifiable by NMR of the soluble Cx43CT fragment (28, 31). This discrepancy may result from the differences in the microenvironment of the soluble fragment versus that of the same region when it is part of the native Cx43 and is integrated into the lipid bilayer. In the former, this segment would be flexible, whereas in the latter its associations would provide it with a more rigid conformation. We speculate that in our experimental conditions, the “floppy,” more flexible amino end may decrease the affinity of regions 281—295 and 299—304 for dimerization, whereas its absence may facilitate the formation of the dimer. Interestingly, Cx43CT residues Ser-314—Ile-327 behave as a rigid structure (i.e., one conformation) in all of the amino-terminal truncations,3 suggesting a strong binding affinity for this dimerization domain. Studies performed in functional channels support the hypothesis that Cx43CT residues Met-281—Asn-295 and Arg-299—Gln-304 are involved in dimerization. These studies demonstrated that deletion of amino acids Met-281—Asn-300 or introduction of a peptide (Cx43CT residues Met-281—Asn-300) into the intracellular space of Cx43-expressing cells renders Cx43 channels less sensitive to acidification-induced uncoupling (34, 35). According to this rationale, deletion or interference with the function of Met-281—Asn-300 would prevent dimerization and, consequently, decrease the ability of Cx43CT to bind Cx43CL. The latter, in turn, would prevent the pH gating process.

The only previously identified dimerization domain that did not alter Cx43CT dimerization when deleted was Asp-340—Ala-348. Two possible explanations for this observation are as follows. 1) This domain is not directly involved in dimerization, and the resonances identified from the filtered nuclear Overhauser effect experiment were intramolecular interactions (24), or 2) association of these residues is inherently weak and transient, and detection of a decrease in dimerization is below the resolution of the cross-linking experiment. Future studies using a more native-like Cx43CT construct where the fourth transmembrane domain is preserved will help us understand whether the behavior of this region (and the others involved in dimerization) is significantly modified by their proximity to the lipid environment of the cell membrane.

It is important to note that under the experimental conditions used to determine the oligomerization state of the soluble Cx43CT by sedimentation equilibrium, the best fit of the data were the monomer-dimer-trimer model, not the monomer-dimer or monomer-dimer-tetramer models (24). Although the trimer percentage was minimal (~1%), this conformation was consistently seen on gels from the chemical cross-linking of the Cx43CT wild type (Figs. 2B, pH 5.8, and 5B, lanes 2 and 3). Therefore, the question arises as to what is the potential oligomerization state in the context of a functional gap junction channel. Using the radius of a connexon of 35 Å and a Cx43CT height of 100 Å (33), this places the effective local concentration of 6 Cx43CT particles at the mouth of an assembled connexon to be ~25 mM. Taken alone, this would push the percent dimerization to 99.9% (Cx43CT/Cx43CT $K_D = 0.21 \mu M$); however, the fact that these CTs are not free in solution but oriented with fixed positions due to their attachment to the membrane would suggest the trimer conformation (i.e., a “dimer of trimer”) cannot be ruled out as a possible physiologically significant quaternary structure. Similarly, we estimate that the concentration of the Cx43CL in the microenvironment of the channel is ~125 mM (radius, 35 Å; height, 20 Å), which is several orders of magnitude higher than the estimated $K_D$ (0.38 μM). Thus, even though the dominant oligomeric state observed for Cx43CL,$_100_{-155}$ was that of a dimer, the possibility of a trimeric state within the microenvironment of the functional channel cannot be ruled out.

**Significance of Cx43CT and Cx43CL Oligomerization—**Our previous studies (24) and those of Seki et al. (23) would suggest that there is an interaction (even if weak) between Cx43CT and Cx43CL domains at normal pH. We have proposed that these weak interactions are responsible for the “residual” state of the channel at normal pH and that the affinity between these domains is affected by the microenvironment (e.g., pH and phosphorylation state). Accordingly, acidification would increase the Cx43CT/Cx43CL binding affinity by inducing structural modifications (e.g., oligomerization) that bring the channel to the “closed” state.

Our data indicate that solvent acidification leads to homooligomerization of the Cx43CT and Cx43CL domains as well as to a heterodomain (Cx43CT/Cx43CL) interaction. It is possible that oligomerization precedes (and in fact may be needed for) the binding of the particle (CT) to its receptor (CL). Consistent with this idea, the only Cx43CT mutant that failed to dimerize, Cx43CT $\Delta_{315-382}$, encompasses all of the Cx43CT residues (Asn-312—Ile-382) affected by the interaction with the Cx43CL as well as residues that are thought to be necessary for pH gating (34, 35). Overall, we propose that oligomerization of the Cx43CT and Cx43CL domains may be one of the structural changes involved in the pH regulation of Cx43. Whether the Cx43CT/Cx43CL interaction leads to changes in the structure
of the pore-forming regions of the Cx43 protein remains to be determined. Finally, the change in oligomeric state may also play a role in transducing signals from the cell surface to the nucleus (36) by changing which cellular partners can bind the Cx43CT domain.

Functional Significance of the Cx43CT/Cx43CL Interaction—We have previously reported that Cx43CT interacts with a peptide corresponding to the second half of the cytoplasmic loop (amino acids 119–144) (22). However, the stoichiometry of the interaction and the identity of the Cx43CT residues involved were not addressed in our previous study. Here, we chose to use a longer construct (amino acids 100–155) to better maintain the functional integrity of the Cx43CL domain. Our cross-linking experiments showed that Cx43CL100–155 binds to two Cx43CTs, whereas a 1:1 association was observed for the shorter Cx43CT119–144 peptide. Consistent with this observation, the kinetics of Cx43CT/Cx43CL100–155 binding were not described by first-rate order equations (see Fig. 5, C and D); we also noted that the chemical shift changes in the amino acids within the loop domain were larger when the longer construct was used (i.e. Cx43CL100–155). Possible factors contributing to the poor model-fitting by SPR are the oligomerization of the Cx43CT and Cx43CL100–155 domains and multiple Cx43CL100–155 binding sites on the Cx43CT with the potential for multiple stoichiometries, each with different binding affinities. Altogether, these results suggest that, whereas a peptide of region 100–118 does not directly bind to Cx43CT (22), the presence of this sequence (and that of residues 145–155) in the entire construct participates directly or indirectly in a complex interaction that involves more than a single ligand species interfacing with a single binding site.

Our NMR studies demonstrated that the addition of the Cx43CT100–155 polypeptide had significant effects on the position of resonance peaks corresponding to amino acids Arg-376—Asp-379 and Asn-343—Lys-346 of Cx43CT, with additional minor shifts in the resonance peaks between residues Asn-312—Ala-332, Phe-335—Asp-340, Val-347—Ala-349, Ala-357—Gln-361, Ser-364—Arg-374, and Leu-380—Leu-382. A correlation exists between the Cx43CT residues affected by the association with the Cx43CL and the Cx43CT residues involved in dimerization and secondary structure (Fig. 11). Our studies suggest interdependence between these events and a possible mechanism for channel regulation during chemical gating in which formation of the higher order Cx43CT and Cx43CL dimeric structures are necessary for their interaction. Previous studies have illustrated that the extent of the shift in the resonance peaks of a protein upon the addition of a ligand can be indicative of whether the shift is caused by direct binding or indirect, distal conformational changes (37, 38). As such, the large chemical shift changes for Cx43CT residues Arg-376—Asp-379 and Asn-343—Lys-346 are suggestive of the location for the direct interaction with the Cx43CL, whereas the other affected residues may be involved in indirect conformational changes associated with binding at the primary sites. This is interesting from the standpoint of how far the Cx43CT domain has to bend or re-arrange to interact with the Cx43CL. We may speculate that the Cx43CT residues affected by the Cx43CL interaction and present within the Asn-312—Ile-382 region constitute the “ball,” whereas the unaffected residues Ser-255—Ala-311 represent the “chain,” in an analogy with the ball-and-chain model of voltage-dependent N-type inactivation of Shaker (39) and of other K+ channels (40, 41).

Our studies show that Cx43CT residues Arg-376—Asp-379 were affected by both the Cx43CL and the second PDZ domain of ZO-1 (28, 42). This dual function would suggest that the Arg-376—Asp-379 region can act as a regulatory switch that “toggles” between molecular partners to regulate channel function. In previous studies we showed that the Cx43CT association to the SH3 domain of Src disrupts the Cx43CT/ZO-1 interaction. We further showed that the Cx43CT/Src association is enhanced by acidification of the intracellular space (43). This would suggest that the Src-induced disruption of the Cx43CT/ZO-1 association would allow for the interaction of the Cx43CT domain with its intramolecular receptor (the Cx43CL domain), hence leading to channel closure.

Recently, we used phage display to identify novel peptide sequences that can bind to Cx43CT and affect function (44). Our studies led to the discovery of a particular peptide (dubbed RXP-E) that interfered with chemical regulation of Cx43. NMR analysis showed that RXP-E leads to shifts in the resonance peaks of the same amino acids here reported to be largely affected by the presence of Cx43CL. Overall, the data would suggest that RXP-E can compete for the same binding site as Cx43CL, thus preventing pH gating. Future studies will be needed to address this interesting possibility.

In summary, the work described here has enabled us to further understand pH-dependent changes in the structure of Cx43. These studies provide evidence that structural modification of both the Cx43CT and Cx43CL domains are necessary for their association and may play a role in the regulation of gap junctions in response to low pH. The observation that the Cx43CT dimerization domains overlap with the residues involved with the Cx43CL interaction opens the possibility that dimerization may be a structural component of the regulation of gap junctions. Future studies will be directed at determining the kinetics of the Cx43CT/Cx43CL interaction and if Cx43CT dimerization is involved in pH gating under conditions of a functional channel.
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