Asparagine 175 of Connexin32 Is a Critical Residue for Docking and Forming Functional Heterotypic Gap Junction Channels with Connexin26

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The gap junction channel is formed by proper docking of two hemichannels. Depending on the connexin(s) in the hemichannels, homotypic and heterotypic gap junction channels can be formed. Previous studies suggest that the extracellular loop 2 (E2) is an important molecular domain for heterotypic compatibility. Based on the crystal structure of the Cx26 gap junction channel and homology models of heterotypic channels, we analyzed docking selectivity for several hemichannel pairs and found that the hydrogen bonds between E2 domains are conserved in a group of heterotypically compatible hemichannels, including Cx26 and Cx32 hemichannels. According to our model analysis, Cx32N175Y mutant destroys three hydrogen bonds in the E2-E2 interactions due to steric hindrance at the heterotypic docking interface, which makes it unlikely to dock with the Cx26 hemichannel properly. Our experimental data showed that Cx26-red fluorescent protein (RFP) and Cx32-GFP were able to traffic to cell-cell interfaces forming gap junction plaques and functional channels in transfected HeLa/N2A cells. However, Cx32N175Y-GFP exhibited mostly intracellular distribution and was occasionally observed in cell-cell junctions. Double patch clamp analysis demonstrated that Cx32N175Y did not form functional homotypic channels, and dye uptake assay indicated that Cx32N175Y could form hemichannels on the cell surface similar to wild-type Cx32. When Cx32N175Y-GFP- and Cx26-RFP-transfected cells were co-cultured, no colocalization was found at the cell-cell junctions between Cx32N175Y-GFP- and Cx26-RFP-expressing cells; also, no functional Cx32N175Y-GFP/Cx26-RFP heterotypic channels were identified. Both our modeling and experimental data suggest that Asn175 of Cx32 is a critical residue for heterotypic docking and functional gap junction channel formation between the Cx32 and Cx26 hemichannels.

Communication between cells is important for tissue and organ homeostasis in multicellular organisms. Direct intercellular signaling is mediated by gap junction channels composed of connexins (Cxs). These channels cluster in specialized regions of the plasma membrane, which allow passage of ions and small molecules, including second messengers, amino acids, nucleotides, and other metabolites, between adjacent cells (1, 2). Gap junctions play crucial roles in many biological processes, including development, differentiation, cell synchronization, neuronal activity, and immune responses (3–5). Mutations in connexins cause a number of human disorders, including deafness, cataract, skin diseases, cardiac defects, neurodegenerative diseases, and developmental abnormalities (6–8). To date, 21 different connexins are identified in the human genome and categorized into α, β, and γ isoforms based on sequence homology.

Sequence alignment of all Cxs indicates that they all share similar topology with four transmembrane domains, two extracellular loops (E1 and E2), and one intracellular loop with both amino terminus and carboxyl terminus in the cytoplasmic side. Gap junction channels are formed by end-to-end docking of two hemichannels, each composed of six connexin subunits (9). The connexin composition of gap junction channels defines their unique properties, such as selectivity for small molecules, voltage-dependent gating, and regulation by Ca2+, pH, and/or phosphorylation (4, 8, 9).

Six identical connexins oligomerize to form a homomeric hemichannel, which is able to dock with a hemichannel of the same connexin or a hemichannel of a different connexin to form a homomeric homotypic or homomeric heterotypic gap junction channel, respectively. Although under experimental conditions, many connexins showed the ability to form heterotypic channels with different connexins (9); there is cumulative...
others have suggested that the two cellular loops are essential for hemichannel docking (15, 16); bonds between sulfur and oxygen atoms, 3.7 Å; and hydrogen hydrogen bonds between nitrogen atoms, 3.5 Å; hydrogen hydrogen bonds between oxygen and nitrogen atoms, 3.5 Å; were as follows: ionic bonds between oxygen and nitrogen code 2ZW3) (16, 22). The threshold values for the interactions previously determined at 3.5 Å resolution (Protein Data Bank junction channels that stabilize the channel structure were as compatible Cxs, suggesting that the docking is selective (9, 14). Functional studies in paired Xenopus oocytes and transfected cell lines expressing different connexins demonstrate that heterotypic gap junction channels can be formed only between compatible Cxs, suggesting that the docking is selective (9, 14). Some works have indicated that the two β-strands at the extracellular loops are essential for hemichannel docking (15, 16); others have suggested that the two β-strands at the extracellular loops might be important in forming proper tertiary structure of the connexin monomer but do not directly affect hemichannel docking (17, 18). In addition, the use of a chimera approach to switch domains of one Cx with the corresponding domains of another Cx has revealed that E2 is critical for heterotypic compatibility (19–21), and E1 appears to be involved in forming part of the channel pore (17). Because the tertiary structure data were lacking, point mutation studies alone were not able to yield enough insights; the molecular structural basis underlying hemichannel docking selectivity remains to be elucidated.

We have reported the structure of the human Cx26 homomeric homotypic gap junction channel (16, 22). This high resolution structure of the Cx26 channel provides an excellent template for us to develop homology structural models for other Cxs. Here we combine the structural modeling with functional study using double patch clamp and confocal imaging to decipher the molecular basis for heterotypic docking between Cx32 and Cx26 hemichannels. Our results indicate that hydrogen bonds (Hb)s involving asparagine 175 of Cx32 at the docking interface are critical in forming functional heterotypic gap junction channels with Cx26.

EXPERIMENTAL PROCEDURES

Analysis of Interhemichannel Interactions in the Cx26 Gap Junction Channel—Non-covalent interactions between two hemichannels that stabilize the channel structure were inspected using the program CONTACT from the CCP4 package (23). The structure of the Cx26 gap junction channel was previously determined at 3.5 Å resolution (Protein Data Bank code 2ZW3) (16, 22). The threshold values for the interactions were as follows: ionic bonds between oxygen and nitrogen atoms, 3.4 Å; hydrogen bonds between oxygen atoms, 3.4 Å; hydrogen bonds between oxygen and nitrogen atoms, 3.5 Å; hydrogen bonds between nitrogen atoms, 3.5 Å; hydrogen bonds between sulfur and oxygen atoms, 3.7 Å; and hydrogen bonds between sulfur and nitrogen atoms, 3.7 Å.

Sequence Alignments—We performed multiple sequence alignments for homology modeling and analysis using the program ClustalW2 from the EBI package (24). We selected Cx26, Cx32, Cx37, Cx43, and Cx50, which have been studied for compatibility in forming functional homomeric heterotypic gap junction channels. Sequence identities of these connexins against Cx26 were higher than 50%, and similarity is more than 80%, and the lengths of the E1 and E2 are generally the same among these connexins. Consequently, Cx26 is an appropriate three-dimensional template for homology modeling for these connexins. According to heterotypic compatibility to form functional gap junction channels, Cx26, Cx32, and Cx50 are classified as Group I, and Cx37 and Cx43 are classified as Group II (9, 14).

Construction of Gap Junction Channel Homology Models and Estimating Accuracy of Models—We generated models of heterotypic and homotypic gap junction channels using all of the selected connexin combinations. Initial models were built using Coot (25), in which the amino acid residues of a Cx26 hemichannel were replaced with the equivalent residues of each connexin based on the sequence alignments, and then we optimized the position of each side chain on Cx26 structure. Next, one hemichannel was merged with an opposing hemichannel using the program PDBSET from the CCP4 package (26), resulting in a heterotypic gap junction channel. Finally, the molecular structures were refined by energy minimization without any structure factor term using the program model to minimize in the CNS program (27). In modeling, we selected space group P1 (originally C2) to remove any effects of crystallographic symmetry. The unit cell parameter, non-crystallographic symmetry was calculated using Cx26 itself, and other CNS settings were default. The quality and stereochemistry of the predicted models of heterotypic channels were evaluated using the programs PROCHECK and SUPERPOSE of CCP4 (26, 28). All molecular representations were created using PyMOL (29).

Cell Culture and Reagents—All cell culture media, sera, and other reagents were obtained from Invitrogen, BD Biosciences, or Sigma. The mouse neuroblastoma (N2A) cells and the human cervical carcinoma (HeLa) cells were purchased from ATCC (American Type Culture Collection) and cultured as reported previously (30, 31).

Engineering of Connexin Chimeric and Mutant Cx32 cDNAs—The Cx26-RFP construct was generously provided by Dr. Dale Laird (University of Western Ontario, London, Canada). To generate the Cx32-GFP fusion protein, the cDNA of the human Cx32 was amplified by PCR with the forward primer (5′-CCT AGC TCG AGA TGA ACT GGA CAG GTT TGT ACA C) to introduce the Xhol site and the reverse primer (5′-TCG GCC TGC TGG CAT CCA ACA G) to introduce the BamHI site. PCR products were digested with Xhol/BamHI and cloned into the Xhol/BamHI-cut pEGFP-N1 (Clontech) vector. DNA sequence analysis confirmed that Cx32 was fused in-frame to the amino terminus of GFP with the addition of an amino acid linker encoded by TGG GAT CCA GCC GTC. The Cx32N175Y construct was used for transient transfection—Cells were transfected with pEGFP- or RFP-tagged connexins by Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer, as described previously (33, 34). In heterotypic compatibility studies, cDNAs of wild-type/mutant Cx32-GFP and Cx26-RFP were individually transfected into N2A or HeLa cells. 24 h after transient transfection, expression levels were examined under fluorescent micro-
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scope. Then wild-type or mutant Cx32-GFP-expressing cells were mixed with Cx26-RFP-expressing cells at a 1:1 ratio. 24 h after mixing, these cells were used for patch clamp recording or fixed for confocal imaging.

Confocal Imaging—After nucleus staining with Hoechst, fixed cells were imaged on a Zeiss (Thornwood, NY) LSM 510 META confocal microscope mounted on an inverted Axiovert 200 motorized microscope equipped with a ×63 oil (1.4 numerical aperture) objective. Fluorescence signals were imaged after excitation with 488-, 543-, or 730-nm laser lines produced by argon, helium/neon, or tunable Chameleon multichip lasers, respectively. All fluorescence signals were collected on a photomultiplier after a passage through appropriate filter sets (33).

Patch Clamp Electrophysiology—Gap junctional coupling between paired N2A cells expressing fluorescent protein-tagged connexins was assessed using the dual whole-cell patch clamp technique as described previously (33, 34). In homotypic configuration, isolated cell pairs with green or red fluorescent gap junction plaques at cell-cell contacts were chosen for patch clamp recording. In heterotypic compatibility studies, only cell pairs of one red fluorescent cell and one green fluorescent cell in intimate contact were selected for recording. Gap junctional conductance (G\textsubscript{j}) was calculated and presented as mean ± S.E. On-line series resistance compensation at 80% or off-line series resistance compensation (35) was applied to improve the accuracy of measured G\textsubscript{j}.

Dye Uptake Assay—The plasma membrane-impermeable fluorescent dye, propidium iodide (PI; Invitrogen), was used in a dye uptake assay to test hemichannel permeability. HeLa cells were incubated for 5 min in Ca\textsuperscript{2+}-free Hanks’ balanced salt solution (HBSS; Invitrogen) and subsequently exposed to 0.15 mmol/liter PI in HBSS with zero Ca\textsuperscript{2+} for 10 min. Cells were then washed three times with HBSS containing 3 mmol/liter Ca\textsuperscript{2+} before being examined under a fluorescence microscope. For each group, 10 fields were randomly selected from three transfections, and only transfected single cells (as determined by the presence of GFP, except control) were counted for the dye loading percentage calculation (36).

RESULTS

Homomeric Heterotypic Model for Cx26/Cx32 Gap Junction Channel—All human connexin genes have very high sequence homology against human Cx26. Especially for Cx32, the protein sequence identity between Cx26 and Cx32 is about 69% in their entire sequence alignment, which is sufficient for generating a homology structural model. The initial model of the Cx32 homomeric hemichannel was formed by using the coordinate of the crystallized Cx26 gap junction channel (16). Based on sequence alignment, residues in the Cx26 Protein Data Bank file (2ZW3) were replaced with the corresponding residues in the Cx32 sequence. Also based on the crystal structure of Cx26, two hemichannels of the model were generated. Finally, the two hemichannels from Cx26 and Cx32 were docked into a whole gap junction channel and minimized in energy terms (for details, see the homology model PDB file in the supplemental material).

Fig. 1A shows our model for a homomeric heterotypic gap junction channel of Cx26/Cx32. A top view of the docking region is displayed in Fig. 1B, and a side view of one pair of interdocked Cx26/Cx32 at the docking region is illustrated in Fig. 1C. This heterotypic gap junction model indicates that Cx26 E1 interacts with Cx32 E1, forming part of the gap junction channel pore lining region, and HBs are formed at the E1-E1 docking interface (shown as dotted lines in Fig. 1C). On the other hand, Cx26 E2 interacts with Cx32 E2 on the exterior of the gap junction channel, forming the extracellular wall of the channel between two cell membranes (Fig. 1, B and C). At the E2-E2 interface between a pair of docked Cx26 and Cx32, six HBs are formed to link these two interdocked Cxs (Fig. 1C; for details, see below).

Docking Interactions at the Interface of the Cx26/Cx32 Heterotypic Gap Junction Channel—A closer look at the E1-E1 docking interface of the Cx26/Cx32 heterotypic channel model reveals that Cx26Q57 forms two HBs with Cx32Q57. Cx26N54 forms one HB with the main-chain amide of opposite Cx32L56, and inversely, Cx32N54 forms a HB with the main-chain amide of the opposite Cx26L56 (Fig. 2A). On average, there are four HBs between each pair of interdocked Cx26/Cx32. It is noted that one E1 of Cx26 interacts with two different Cx32 subunit E1 domains, and vice versa (Fig. 2A, bottom). This is why, in Fig. 1C, only two HBs are visible in one pair of docked Cx26/Cx32 subunits.

FIGURE 1. Overall structure and interhemichannel interactions of the Cx26/Cx32 heterotypic gap junction channel. A, side view of a whole Cx26/Cx32 heterotypic gap junction channel model. The main peptide chain Cα trace is shown as a ribbon and line diagram, with the Cx26 hemichannel on the top (one of the subunits is highlighted in magenta) and the Cx32 hemichannel at the bottom (one of the subunits is highlighted in green). B, a top view of the docking interface region of the Cx26/Cx32 heterotypic channel. The Cα trace is shown as a line diagram. E1 forms the pore lining region (light blue), and E2 is located on the outer part of the channel (purple). C, a closer view of docking interactions at the docking region between a pair of Cx26 (magenta) and Cx32 (green) protomers. The dotted lines represent hydrogen bonds at the docking interface.
At the E2-E2 docking interface in the Cx26/Cx32 heterotypic channel, our model predicts a total of six interconnexin HBs in each pair of docked Cx26 and Cx32 (Fig. 2B). All of the residues for these interconnexin HBs are illustrated in Fig. 2B. Two key residues are Cx32N175 and Cx26N176, each of which contributes three HBs between a docked pair (Fig. 2B). Specifically, Cx32N175 forms HBs with Cx26 Lys168, Thr177, and Asp179. One of these HBs is with the main-chain carbonyl groups of Thr177, and the other two are between amino acid side chains (Fig. 2B, bottom). Inversely Cx26N176 develops HBs with Cx32 Lys167, Thr176, and Asp178. These heterotypic HB interactions at the docking interface are the same as those in Cx26 homotypic channel. It is interesting to note that, distinct from E1-E1 HB interactions, the E2-E2 HB interactions are within the same pair of docked Cx26/Cx32 subunits (Fig. 2B).

**Heterotypic Docking Compatibility Appears to Depend on the Interactions at E2-E2, Not Those at E1-E1**—To study the docking compatibility for homomorphic heterotypic gap junction channel among different Cxs, we selected five well studied Cxs for our model analysis. Compatibility data for heterotypic pairing between any two of the five Cxs are summarized with only one unknown (Fig. 3A). According to their heterotypic compatibility, these Cxs are classified into Group I (Cx26, Cx32, and Cx50) and Group II (Cx37 and Cx43). Cxs in the same group are compatible to form functional heterotypic gap junction channels, but not intergroup Cxs (yellow boxes). The table is modified from Yeager and Nicholson (14) and Harris (9). B, sequence alignment of selected Cxs at E1 (corresponding to amino acid residues 44–72 of Cx26) and E2 (corresponding to amino acid residues 160–185 of Cx26) regions. The secondary structure is shown on the top. The cylinders represent α helices, and the ribbons with arrows represent β strands. HB-forming residues are highlighted with arrows and blue shading (using their side chains) or arrows only (using the amide/carbonyl main chain). Color coding of amino acid residues is as follows. Orange, residues strictly conserved among human connexins; green, polar residues; red, acidic residues; blue, basic residues; purple, hydrophobic residues; yellow, aromatic residues.

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Fig. 3B. Based on the high sequence homology of the selected Cxs with Cx26 at E1 and E2 domains, these Cxs are likely to share similar structure to that of the Cx26 gap junction channel at the docking interface. We generated homology models to analyze the heterotypic docking compatibility between intragroup and intergroup Cx pairs. Our models indicate that heterotypic E1-E1 interactions are almost identical for both intragroup and intergroup Cx pairs. They can form hydrogen bonds similar to those in Cx26/Cx32 heterotypic channel. The key amino acid residues forming hydrogen bonds (Gln$^{157}$ of Cx26 or equivalent and Asn$^{154}$ or equivalent) are identical for all selected Cxs. The main-chain amide at Cx26L56 is not affected by different amino acid residue side chains for other Cxs. Importantly, no major structural differences for E1-E1 interactions between inter- and intragroup Cx pairs were identified. Thus, our model predicts that E1-E1 interaction is unlikely to be the key component in determining heterotypic docking compatibility.

On the other hand, our models on heterotypic E2-E2 interactions showed that one HB forming residue Asp$^{179}$ of Cx26 (or equivalent) is conserved for all selected Cxs. The main-chain carbonyl group of Thr$^{177}$ of Cx26 (or equivalent) was predicted to be involved in forming another HB. Because the HB is on the main peptide chain, not the amino acid side chain, it is unlikely to be affected by the different amino acid residue at this position.
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(Thr, Val, Leu, or Gln) in the selected Cxs. Hence, our model analysis predicted that Thr<sup>177</sup> and Asp<sup>179</sup> on E2 are unlikely to be the key residues for heterotypic compatibility. However, the major HB-forming residue, Asn<sup>176</sup> of Cx26 (equivalent to Asn<sup>175</sup> of Cx32), is completely conserved in all Group I Cxs, but in Group II at the same position, one finds a much larger amino acid residue, Tyr (Cx37) or His (Cx43) (see Fig. 3B). Interestingly, Lys<sup>168</sup> of Cx26 is also conserved in Group I Cxs, with Lys for Cx32 and Arg for Cx50, both of which are positively charged and relatively bulky residues. But at the corresponding position for Group II Cxs, one finds a much smaller amino acid residue, Val for Cx37 or Thr for Cx43 (see Fig. 3B). Based on the sequence homology and our models, it appears that Asn<sup>176</sup> and Lys<sup>168</sup> of Cx26 (equivalent to Asn<sup>175</sup> and Lys<sup>167</sup> in Cx32) are the key residues for docking compatibility in forming functional heterotypic gap junction channels.

To start experimentally testing the roles of E2-E2 HB interactions in heterotypic docking compatibility, we chose to create the Cx32N175Y mutant (equivalent to Asn<sup>176</sup> of Cx26) to see if this mutated Cx32 was able to dock with Cx26 to form a functional heterotypic gap junction channel. Our homology model predicts that, with this mutation, three HBs between the E2 of Cx32 and the E2 of Cx26 are destroyed (Fig. 4, A and B). Tyrosine in the 175 position of Cx32 (Cx32N175Y) is so large that it collided with Lys<sup>168</sup> of Cx26 and removed all three HBs after spatial optimization.

**Cx32N175Y Exhibited an Intracellular Distribution Pattern and Failed to Form Functional Gap Junction Channels**—Cx26 and Cx32 gap junction channels have been extensively studied in *Xenopus* oocytes or mammalian cells, mostly in untagged forms. When transfected into HeLa and N2A cells, our constructs of Cx26-RFP and Cx32-GFP were able to traffic to the plasma membrane and form gap junction plaques at cell-cell interfaces with some punctate intracellular distribution (Fig. 5, A and B). According to double patch clamp recordings, Cx26-RFP- and Cx32-GFP-expressing cells presented robust homotypic gap junctional coupling and the characteristics of Cx26 and Cx32 channel gating, respectively (Fig. 5D). All of these observations suggest that tagging red/green fluorescent proteins to the carboxyl terminals of Cx26 and Cx32, respectively, does not alter their expression, trafficking, and major gap junction channel properties.

However, when expressed in N2A and HeLa cells, Cx32N175Y-GFP exerted predominant cytoplasmic distribution, although fluorescent plaques could be seen occasionally at cell-cell junctions (Fig. 5C). Moreover, Cx32N175Y-GFP did not form functional gap junction channels, even in some cell pairs with plaque-like structures at cell-cell interfaces (Fig. 5, C and D).

**Cx32N175Y- and Wild-type Cx32-expressing Cells Showed Similar Propidium Iodide Uptake**—To confirm that the Cx32N175Y mutant could traffic to the cell surface to form functional hemichannels, the PI uptake assay was applied to HeLa cells transfected with mutant and wild-type Cx32-GFPs. To eliminate the possible interference from gap junction channels, only GFP-positive (except control) single cells isolated from other cells were chosen to check PI loading effect. In zero Ca<sup>2+</sup> HBSS, PI was able to permeate into mutant and wild-type Cx32-expressing cells but not control cells (Fig. 6A). The summarized results showed that less than 10% of the control HeLa cells picked up PI dye, which could be accounted for background, and all of the mutant and wild-type Cx32-expressing cells counted were loaded with the fluorescent dye (Fig. 6B). These results indicated that the Cx32N175Y mutant was able to form functional hemichannels on the cell surface not different from those formed by wild-type Cx32 in terms of the fluorescent dye uptake.

**FIGURE 4.** E2-E2 interactions at the docking interface between Cx26 and Cx32 (or Cx32N175Y). Shown are the interactions at the docking interface between Cx26 (magenta) and Cx32 (green). Six HBs are formed in each pair of docked Cx26/Cx32 (A). Cx32N175Y mutation causes spatial conflict at the docking interface (B). After optimization of spatial orientation, three HBs were eliminated (C). In Cx32N175Y/Cx32N175Y homotypic channels, the HBs between the interdocked Cx pair were completely eliminated (D). One-letter amino acid codes are used.
Cx32N175Y Was Not Able to Form Heterotypic Gap Junction Plaques with Cx26—To visually examine heterotypic compatibility, Cx26-RFP, Cx32-GFP, and Cx32N175Y-GFP were separately transfected into HeLa and N2A cells. After 24 h, cells were suspended and then intermixed in an attempt to form two heterotypic pairs between Cx26-RFP and Cx32-GFP or Cx26-RFP and Cx32N175Y-GFP. High resolution confocal imaging revealed that when a Cx26-RFP-expressing cell had intimate contact with a Cx32-GFP-expressing cell, co-localization of red and green fluorescence at the cell-cell interface was frequently seen (Fig. 7, arrow in left column). On the other hand, Cx32N175Y-GFP did not form mixed plaques with Cx26-RFP at the cell-cell junction (Fig. 7, right column).

Cx32N175Y Was Unable to Form Functional Heterotypic Gap Junction Channels with Cx26—The double patch clamp technique was also applied to test the heterotypic gap junction channel formation. Under a fluorescent microscope, only cell pairs of one green cell and one red cell with intimate contacts were selected for patch clamp recordings. As expected, Cx32-GFP- and Cx26-RFP-expressing cell pairs were able to form functional homotypic channel, whereas N2A cell pairs expressing Cx26-RFP or Cx32-GFP exerted robust electrical coupling (Fig. 8). The total number of recorded cell pairs is indicated above each bar. Error bars, S.E.
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FIGURE 7. Cx32N175Y does not co-localize with Cx26-RFP in cell junctions. When Cx32-GFP expressing HeLa cells had intimate contacts with Cx26-RFP-expressing cells, the co-localization of green and red fluorescent plaques was often seen at cell-cell interfaces, forming yellow overlay plaques (left column, arrow). However, Cx32N175Y-GFP appeared not to co-localize with Cx26-RFP at cell-cell junctions (right column). Scale bars, 10 μm.

FIGURE 8. Cx32N175Y was not able to form functional heterotypic channel with Cx26-RFP. When Cx32-GFP-expressing N2A cells were mixed with Cx26-RFP-expressing cells, electrical coupling was established between one green fluorescent cell and one red fluorescent cell. An asymmetric voltage gating profile was observed, which is characteristic of Cx32/Cx26 heterotypic gap junction channels (A, left). On the other hand, Cx32N175Y-GFP was not able to form a functional heterotypic channel with Cx26 (A, right). Quantitative coupling conductance was measured at a 20-mV voltage step and is shown as a bar graph (B). The total number of recorded cell pairs is indicated above each bar. Error bars, S.E.

Although the structure in the extracellular domains was poorly resolved in an early structural model (39), functional studies with domain swapping connexin chimeras pointed to E2 as a determinant for hemichannel docking selectivity (19, 21). Dahl \textit{et al.} (40) used a series of peptides derived against the two extracellular loops of Cx32 to inhibit cell-cell channel formation, and they were able to narrow down several segments in E1 and E2 of Cx32 as critical stretches for docking and functional channel formation. In a delicate tentative model on docking interface structure of Cx32, Nicholson and colleagues (15) described an antiparallel double β barrel structure formed by interdigitated E1 and E2 domains from end-to-end hemichannels. Several works also suggested that the conserved cysteine residues in the extracellular loops could only form intracconnexin disulfide bonds (15, 41, 42), indicating that the exchange of disulfide bonds should not directly contribute to hemichannel docking rather stabilizes already docked channels. Our knowledge about the interactions involved in end-to-end hemichannel docking was first obtained from the attempts to chemically split gap junctions in order to perform deep-etch freeze fracture. Subsequently, atomic force microscopy was applied to probe the surface structure of fractured junctional membrane containing hemichannels (9, 43). According to the minimum conditions required to split isolated gap junctions, it was speculated that hydrogen bonding might be the stabilizing factor for junctional structures (9, 41). Moreover, it was easily noticed that the sequences of E1 and E2 domains are highly rich in hydrophobic residues; hence, hydrophobic interactions might be important in maintaining the tertiary structure of E1 and E2 domains (44).

Previous structural data showed that, under proper docking, two hemichannels are not aligned straight but rather have 30°
rotation (45, 46). Consistently, our structure modeling on Cx26/Cx32 heterotypic channel indicated that, although every E2 domain of one hemichannel only interacts with one E2 domain of the opposing hemichannel, every E1 domain does form hydrogen bonds with two E1 domains of the other hemichannel. Unlike other models describing the structure of the junctional interface, our structural model also suggested a less interdigitated end-to-end docking interface. The concentric double β-barrel structure model did not specify which loop (E1 or E2) is innermost to face the aqueous lumen of the pore (15); subsequent studies using a E1 domain substitution strategy and the substituted cysteine accessibility method demonstrated that the E1 domain was lining the channel pore and determined charge selectivity in Cx46 channels and hemichannels (17, 18). In all of the homotypic and heterotypic channels we modeled in our study, the E1 domains from both hemichannels are predicted to form a tight barrel as the channel inner wall in the gap region between the two cytoplasmic membranes.

Our structure models indicate that a total of 60 HBs are formed between a pair of interdocked Cx32/Cx26 hemichannels; 24 of these HBs are between E1-E1 domains and the remaining 36 HBs are between E2-E2 domains in the heterotypic docking interface. The modeling analysis on two groups of well characterized Cxs suggests that E1-E1 interactions at the docking interface are homogeneous among all of the selected Cxs, whereas the E2-E2 interactions in terms of HBs are clearly distinct between compatible versus non-compatible groups of Cxs.

In a closer examination of the E1 residues contributing to interhemichannel interactions, hydrogen bonds are observed involving Asn175 of Cx32 (or equivalent residues) in all of the homology models and with every combination of connexins as well as Gln57 of Cx26, which is strictly conserved among all human connexins at the corresponding position. In the homology models, all of the E1 loops produced reasonable structures with the same interactions as those observed in the Cx26 gap junction channel. Although some connexins contain a Gln or Ala residue at the position of Leu56, these residue differences will not affect the hydrogen bond formation with Asn54 on the main-chain amide group. These different side chains are predicted to face the pore lumen and are pore lining residues likely to affect channel permeability. Consequently, the E1 loops contribute to the formation of all gap junction channels but do not appear to be a selective determinant for docking partners because there is no major difference in E1 domains between incompatible groups of Cxs.

In the E2 loop of Cx26, Asn176 forms hydrogen bonds with the side chains of Lys168 and Asp179 and the main-chain carbonyl of Thr177 from the opposite Cx26 hemichannel (16). Homology modeling suggested that residues 168 and 176 are key residues for the selective docking and formation of a functional gap junction channel. When Asn176 of Cx26 was replaced with Tyr (mimicking Cx26/Cx37 pairing), the new 176th residue, Tyr176, would collide with Lys168 and all of the interhemichannel hydrogen bonds were lost. Likewise, the Cx32N175Y mutant that mimics Cx37 lost half of the hydrogen bonds when docking with Cx26 in heterotypic configuration, and in the case of the Cx32N175Y homotypic channel, the mutant lost all hydrogen bonds with the opposite hemichannel. If the hydroxyl group of Tyr176 interacts with carbonyl oxygen of Thr177 or Asp179, it would need significant structural changes in the E2 domain. Also, when Asn176 of Cx26 is replaced with His (mimicking Cx26/Cx43 pairing), the His176 might produce an electric repulsion with Lys168 of the docked hemichannel. Furthermore, it would be stereo-geometrically impossible to simultaneously establish interactions between His176 and Asp179 and between His176 and Thr177. In the case with longer and bulkier residues at the 168th and 176th positions in opposing hemichannels, the interactions between the hemichannels are also disrupted by steric hindrance. Thus, connexins with His or Tyr at the 176th residue cannot form heterotypic channels with connexins containing Lys or Arg at the 168th residue. The interhemichannel hydrogen bonds between the 176th and 179th, 168th and 176th, or 177th and 176th residues were also fully or partially eliminated with Tyr or His at the 176th residue. In other words, connexins with bulky amino acids at the 176th residue, such as Tyr or His, can only form homotypic or heterotypic gap junction channels with connexins that have smaller residues at the 168th position.

Cx32 and Cx26 gap junction channel properties have been extensively studied in Xenopus oocytes and mammalian cells. Fluorescent protein-tagged Cx32 and Cx26 were also expressed in various cell lines to monitor their intracellular targeting and functionality (31, 47–50). Although there is still controversy on their trafficking pathways, both fused proteins were able to reach plasma membranes forming gap junction channels that were permeable to fluorescent dyes (31, 47, 49, 50). In current study, we found that our engineered Cx32 and Cx26 constructs fused with GFP and RFP, respectively, readily formed gap junction plaques in cell–cell interfaces when expressed in HeLa and N2A cells; double patch-clamp recordings revealed that these Cx32-GFP and Cx26-RFP homotypic channels are functional and exert characteristics not different from those previously observed in Xenopus oocytes and N2A cells (37, 38). Thus, tagging fluorescent proteins to the carboxyl termini of Cx32 and Cx26 did not appear to change their intracellular targeting and macroscopic gap junction channel properties noticeably.

Connexins tagged with fluorescent proteins of different colors are ideal tools for heterotypic docking studies in mammalian cells. When Cx32-GFP and Cx26-RFP hemichannels were provided with sufficient opportunities for heterotypic interactions, they were able to dock to each other and form gap junction plaques of mixed colors at cell–cell interfaces. Double patch clamp recordings demonstrated that the heterotypic gap junction channels underlying these overlapping plaques were functional. Moreover, detailed gating analysis revealed an asymmetric gating profile that is typical for Cx32/Cx26 heterotypic channels observed previously (37, 38). These rectified gating characteristics were due to the differential charge selectivity of Cx32 and Cx26 hemichannels, respectively. Thus, our data demonstrated that tagging fluorescent proteins to the carboxyl termini of Cx32 and Cx26 did not affect the heterotypic docking and functional channel formation and the characteristics of the transjunctional voltage-dependent gating properties.

Both our homology modeling and previous functional studies point to the 176th residue in Cx26 (or 175th residue in Cx32)
Asn\textsuperscript{175} of Cx32 Is a Critical Residue for Heterotypic Docking

as the most important residue for selective docking; thus, we created the Cx32N175Y mutant to explore its homotypic docking and heterotypic docking with Cx26. Under the homotypic docking configuration of the N175Y mutant, our modeling predicted that Cx32 Lys\textsuperscript{167} has a spatial conflict with Tyr\textsuperscript{175} of the interdocked hemichannel and that all of the hydrogen bonds in E2-E2 interactions between two Cx32N175Y hemichannels are destroyed. Thus, Cx32N175Y homotypic channel docking and functional channel formation should be drastically disturbed. Abundant intracellular distribution of Cx32N175Y-GFP reflected altered trafficking and intracellular targeting; to our surprise, occasional plaque formation was still observed. It is very likely that even without critical hydrogen bonds around N175Y, at least docking and plaque formation could still be initiated, but it cannot be completed to form functional gap junction channels. It has been reported in many cases that gap junction plaque formation and functional channel formation might be separate events. In supporting this notion, we previously observed that several Cx43 mutants linked to oculodentodigital dysplasia could form gap junction plaques but not functional gap junction channels (34).

Although Cx32N175Y homotypic gap junction plaques were occasionally observed, implicating the ability of this mutant to reach the cell surface, we could not rule out the possibility that the failure in forming functional homotypic and heterotypic channels by Cx32N175Y was due to its impaired intracellular trafficking but not defective docking. Because of technical limitations, confocal imaging was not able to reliably visualize connexin distribution in single plasma membranes. The dye uptake results demonstrated that Cx32N175Y was targeted to the cell surface and able to form functional hemichannels similar to wild-type Cx32, indicating that this mutant is indeed impaired in the docking with itself or Cx26.

Several studies with disease-linked mutations in the E1 and E2 regions also shed light on the interactions between extracellular loops. Mutations in the gene encoding Cx32 are found to cause the X-linked form of Charcot-Marie-Tooth disease (13). It is interesting that three key residues in the E2 predicted to be involved in hydrogen bond formation at the docking interface (i.e. Lys\textsuperscript{167}, Asn\textsuperscript{175}, and Asp\textsuperscript{178} of Cx32) are mutation hot spots. X-linked Charcot-Marie-Tooth disease Cx32 mutations, K167E, N175D, and D178Y (51–53), are very likely to impair homotypic and/or heterotypic docking processes, leading to impaired gap junction intercellular communication and disease states in patients. Similarly, nonsyndromic deafness was reported to be associated with a mutation on one of the HB-forming residues at Asp\textsuperscript{179} in the E2 of Cx26 (D179N) (54). More functional studies on these mutants in the E2 domain of these Cxs are in demand to reveal if these mutations are indeed causing docking impairment.

In the present study, we mainly focused on switching the key HB-forming residue (Cx32N175) at the E2-E2 docking interface of Group I Cxs to Tyr, the corresponding residue of Group II member Cx37. It will be of great interest to work on Cx32N175H and other mutations at this position to further explore general docking mechanisms between connexin hemichannels. These future experiments may help us to address the questions of how many HBs and which HB(s) at the E2-E2 docking interface are required for functional docking of heterotypic gap junction channels.

CONCLUSION

We predicted three-dimensional structures of homo- and heterotypic gap junction channels among five human connexins by homology modeling. The hydrogen bonds between the two E1 domains in opposing hemichannels were conserved among all of the docking pairs of the inspected gap junction channels, which serve to stabilize the docked gap junction channel. Our modeling results further showed that Cx32 point mutation of N175Y in the E2 domain destroyed all of the hydrogen bonds when forming a homotypic channel with itself or a heterotypic channel with Cx26, indicating a critical role for this residue in functional gap junction channel docking. The functional studies demonstrated altered intracellular distribution, impaired homotypic docking, and no functional gap junction channel formation for Cx32N175Y mutant. Furthermore, this mutant Cx32 failed to dock (co-localize) with Cx26 in cell-cell junctions to form a functional heterotypic channel. Thus, both structural and experimental data suggested that asparagine 175 of Cx32 is a critical residue for docking and for the formation of functional heterotypic gap junction channels with Cx26.

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