A mechanism of gap junction docking revealed by functional rescue of a human-disease-linked connexin mutant

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Summary
Gap junctions are unique intercellular channels formed by the proper docking of two hemichannels from adjacent cells. Each hemichannel is a hexamer of connexins (Cxs) – the gap junction subunits, which are encoded by 21 homologous genes in the human genome. The docking of two hemichannels to form a functional gap junction channel is only possible between compatible Cxs, but the underlying molecular mechanism is unclear. On the basis of the crystal structure of the Cx26 gap junction, we developed homology models for homotypic and heterotypic channels from Cx32 and/or Cx26; these models predict six hydrogen bonds at the docking interface of each pair of the second extracellular domain (E2). A Cx32 mutation N175H and a human-disease-linked mutant N175D were predicted to lose the majority of the hydrogen bonds at the E2 docking-interface; experimentally both mutations failed to form morphological and functional gap junctions. To restore the lost hydrogen bonds, two complementary Cx26 mutants – K168V and K168A were designed to pair with the Cx32 mutants. When docked with Cx26K168V or K168A, the Cx32N175H mutant was successfully rescued morphologically and functionally in forming gap junction channels, but not Cx32 mutant N175Y. By testing more homotypic and heterotypic Cx32 and/or Cx26 mutant combinations, it is revealed that a minimum of four hydrogen bonds at each E2-docking interface are required for proper docking and functional channel formation between Cx26 and Cx32 hemichannels. Interestingly, the disease-linked Cx32N175D could be rescued by Cx26D179N, which restored five hydrogen bonds at the E2-docking interface. Our findings not only provide a mechanism for gap junction docking for Cx26 and Cx32 hemichannels, but also a potential therapeutic strategy for gap junction channelopathies.

Key words: Gap junction channel, Docking, Dual whole cell patch-clamp, Homology modelling, Hydrogen bond

Introduction
Direct communication between cells is important for tissue and organ homeostasis, and highly coordinated activities in multicellular organisms. In humans and animals, gap junctions are the only intercellular channels that allow the direct exchange of ions and small signaling and metabolic molecules between neighboring cells (Harris, 2001; Kumar and Gilula, 1996; Nicholson, 2003). Gap junctions have crucial roles in many biological processes, including development, tissue and organ synchronization, and immune responses (Levin, 2007; Saez et al., 2003; Sarieddine et al., 2009). Mutations in the genes encoding gap junction subunits, connexins (Cxs), have been associated with a number of inherited human diseases, such as peripheral neurodegenerative disease (X-linked Charcot-Marie-Tooth disease, CMTX), central hypomyelination, non-syndromic hearing loss, catacares, skin diseases and developmental abnormalities (Cottrell and Burt, 2005; Kelsell et al., 2001; Laird, 2010). There are 21 different connexin genes in the human genome. Each connexin displays unique distribution profile, and the gap junction channels formed by different Cxs show distinct biophysical properties (Barrio et al., 1991; Harris, 2001; Nicholson et al., 2000; Simon and Goodenough, 1998).

Functional gap junction channels are formed by the proper docking of two hemichannels (connexons) from adjacent cells, and each hemichannel is a Cx hexamer (Kumar and Gilula, 1996). All Cxs share a common topology with four transmembrane domains connected by two extracellular loops (E1 and E2) and one cytoplasmic loop, leaving both amino and carboxyl termini in the cytoplasm. It has been known for over two decades that the docking of two hemichannels and the formation of functional gap junction channels only occur between compatible Cxs, but the underlying molecular mechanisms are yet to be elucidated. Although lacking detailed insights on the structure of the gap junction docking interface; our knowledge on the process of gap junction docking was largely obtained from the ‘blind’ functional tests, mainly with the paired Xenopus oocyte recording system. As E1 and E2 are the only extracellular domains, they were suggested to be essential for the docking process (Dahl et al., 1994; Foote et al., 1998; Harris, 2001; Pfahnl et al., 1997). Further studies with domain swapping Cx chimeras indicated that the E2 domain is more important for the docking selectivity and formation of functional gap junction channels (White et al., 1994). The crystal structure of human Cx26 gap junction channel model revealed a total of 36
hydrogen bonds at the six E2–E2 docking interfaces (Maeda et al., 2009; Suga et al., 2009), which are predicted to be the same in our homology model of the heterotypic Cx26/Cx32 channel (where the solidus represents a heterotypic complex of Cx26 and Cx32 hemichannels) (Fig. 1). A key hydrogen-bond-forming residue on the E2 domain, that is Cx32N175, was identified in our earlier work. A mutation of this residue, N175Y, resulted in a failure to form homotypic and heterotypic channels probably due to the loss of hydrogen bonds at the E2–E2 docking interface (Nakagawa et al., 2011).

To study further the role of the E2–E2 hydrogen bonds in the docking process and the formation of functional gap junction channels, here we characterized two additional mutants at the same position, Cx32 N175H and N175D; the latter was found to be associated with inherited CMTX (Silander et al., 1998). As predicted, these two mutants also lost the majority of their E2–E2 hydrogen bonds and the function of their homotypic gap junction channel. However, when docked with the designed Cx26 mutant hemichannels, the E2–E2 hydrogen bonds were partially restored and the gap junction function of these mutants could be rescued. By testing different docking pairs of the mutants and/or wild-type Cx32 and Cx26, the experimental data and homology modeling revealed that the function of the gap junction channel of a particular docking pair is dependent on the putative number of the hydrogen bonds at the E2–E2 docking interface. A minimum of four hydrogen bonds at each E2–E2 docking interface are required to establish functional gap junction channels. Our results provide a new docking mechanism for the gap junction channels of Cx32 and Cx26, and probably other compatible connexins.

**Results**

We analyzed 10 human connexins (Fig. 1A), which had been well studied for their heterotypic compatibility (Harris, 2001; Yeager, 2000). These connexins could be separated into two groups: Cx26, Cx30, Cx32, Cx46 and Cx50 form Group 1; and Cx30.3, Cx37, Cx40, Cx43 and Cx45 form Group 2 (Fig. 1A). Generally, the Cxs in the same group are compatible to each other in terms of forming functional heterotypic gap junction channels (Fig. 1A, green areas), but the Cxs from different groups are unable to form functional heterotypic gap junction channels with one another (Fig. 1A yellow area).

In a previous study, we proposed that E1 is unlikely to be the key domain for docking selectivity because the E1 sequence alignment of both compatible and non-compatible Cxs showed high homology, especially at the hydrogen-bond-forming residues (Nakagawa et al., 2011). By contrast, E2 sequence alignment clearly revealed a differential pattern at two of the hydrogen-bond-forming residues between the two groups (black

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**Fig. 1. Human connexin heterotypic compatibility, E2 sequence alignment and a homology structure model of Cx26/Cx32 heterotypic gap junction channel.** (A) Heterotypic compatibility among selected connexins (Cxs). These 10 Cxs are divided into Groups 1 and 2. Generally, functional heterotypic gap junction channels can be formed between intra-group Cxs (green boxes), but not inter-group Cxs (yellow boxes) (modified from Harris, 2001; Yeager, 2000). (B) The second extracellular domain (E2) sequence alignment of the selected Cxs. The E2 domains range from residues 159 to 184 in Cx32, and 160 to 185 in Cx26. The hydrogen-bond-forming residues are highlighted with arrows (modified from Nakagawa et al., 2010). (C) We developed a homology structure model for the wild-type Cx26/Cx32 heterotypic gap junction channel, with the Cx26 hemichannel at the top (magenta) and the Cx32 hemichannel at the bottom (green). One pair of the end-to-end docked Cx26 and Cx32 subunits was highlighted as ribbon and line diagrams; the model predicted six hydrogen bonds at the E2–E2 docking interface (3+3, only one pair of E2–E2 bonds is shown in the inset). The hydrogen-bond-forming residues are: Cx32 N175 with Cx26 K168, T177 and D179; and Cx26 N176 with Cx32 K167, T176 and D178. Note the hydrogen bond within E2 (e.g. between Cx32 K167 and D178, arrow) was not counted as an inter E2–E2 hydrogen bond.
arrows, Fig. 1B). The first one is at the 175th position of Cx32 (or 176 for Cx26), which is an asparagine (Asp or N); the side chain of this residue is involved in forming three inter E2–E2 hydrogen bonds (Fig. 1C). This residue is conserved in all five Cxs in Group 1. The corresponding amino acid residues in Group 2 Cxs are either a histidine (His or H, four out of five) or a tyrosine (Tyr or Y, one out of five) (Fig. 1B), both of which have much bulkier side chains (His with a positive charge and Tyr with an aromatic side chain) compared with that of Asp. The second one is at the 168th position of Cx26 (or 167 for Cx32), which is a lysine (Lys or K). The side chain of Lys is involved in forming one inter E2–E2 hydrogen bond (Fig. 1C). The equivalent residues are highly conserved in Group 1 Cxs, with three out of five residues being Lys and two out of five residues being arginine (Arg or R), both of which have long and positively charged side chains. The corresponding amino acid residues in Group 2 Cxs are also conserved but with small side chains and non-charged residues, such as valine (Val or V), alanine (Ala or A) or threonine (Thr, T) (Fig. 1B). These inter-group discrepancies in the hydrogen-bond-forming residues are the rationales for us to design and generate mutants, and to manage the docking-pairs for structural modeling and functional examination. Thus, we engineered cDNA constructs for Cx32N175Y, Cx32N175H, Cx26K168V and Cx26K168A. In addition, two mutations linked to human disease were also constructed: Cx32N175D was associated with CMTX (Silander et al., 1998); and, interestingly, a structurally complementary mutant Cx26D179N was linked to non-syndromic hearing loss (Primignani et al., 2003).

With these mutants in hand, we adopted three disparate approaches in parallel to tackle the docking mechanisms of Cx32 and Cx26: (1) We developed homology structure models independently for all the mutant and wild-type docking-pairs, in both homotypic and heterotypic configurations, on the basis of the crystal structure of Cx26 to extrapolate the number of hydrogen bonds at the E2–E2 docking interface. In a similar manner to the crystal structure of Cx26 gap junction channel, the homology structural model of wild-type Cx26/Cx32 heterotypic gap junction channel was predicted to have six pairs of E2–E2 docking interfaces, with each interface containing six inter E2–E2 hydrogen bonds, three of them are involved with Cx26N175 and the other three involved with Cx26N176 (see Fig. 1C inset, labeled as ‘3+3’) (Nakagawa et al., 2011). Both inter E2–E2 and intra E2 hydrogen bonds are shown (i.e. those between two docked hemichannels and those within the E2 domain, respectively), but only the inter E2–E2 hydrogen bonds, which are directly related to the docking process, are labeled for each model of the E2–E2 pair structure. (2) Using multiphoton confocal microscopy and fluorescence protein-tagging techniques, the expression of the mutant and wild-type Cxs in gap-junction-deficient HeLa cells as well as their ability of trafficking to cell–cell junctions to form gap junction plaque-like structures were also monitored. The Cx32 (wild type and mutants) and Cx26 (wild type and mutants) were tagged with green and red fluorescent proteins (GFP and RFP, respectively) to facilitate the selection of cell pairs for homotypic or heterotypic configurations. (3) Eventually, the ability to form functional homotypic and/or heterotypic gap junction channels of these mutant and wild-type Cx pairs was investigated by dual whole cell patch-clamp recordings with N2A cells. The gap-junction-coupling conductance (Gj) was measured, and statistical analysis was performed to evaluate the functional status of each docking combination in the formation of gap junction channels.

**Cx32N175H failed to form morphological and functional gap junctions**

Our homology model predicts that the Cx32N175H mutation destroys 24 inter E2–E2 hydrogen bonds at the six docking interfaces for the homotypic mutant gap junction channel. Only two inter E2–E2 hydrogen bonds could be formed for each pair of docked E2 (Fig. 2A), predicting a total of 12 inter E2 hydrogen bonds for each homotypic mutant channel. To evaluate the cellular localization of Cx32N175H in gap-junction-deficient HeLa cells, we tagged GFP at the carboxyl terminus of the mutant to form Cx32N175H–GFP. The tagged mutant displayed a predominant intracellular distribution and was rarely found at the cell–cell junctions (Fig. 2B). Functional tests using dual whole-cell patch clamps in N2A cell pairs expressing wild-type Cx32 (Fig. 2C,D). This suggests that the ability of the mutant to form functional gap junction channels was severely impaired, probably owing to the substantial loss of the inter E2 hydrogen bonds.

**Fig. 2. Homology model, cellular localization and functional status of Cx32N175H.** (A) The homology structure model predicted that the Cx32 mutant N175H lost four out of six hydrogen bonds at each E2–E2 interface. (B) When expressed in HeLa cells, the Cx32 N175H mutant was largely distributed in the cytoplasm and rarely formed gap junction plaque-like structures at cell junctions. Scale bars: 10 μm. (C) Original patch-clamp experiments recorded junctional current traces from wild-type and mutant Cx32 gap junction channels. The lower row shows 20 mV impulse (Vj) applied to one cell, the upper row shows functional status of each docking pair (Gj). (D) Summarizes Gj obtained from mutant or wild-type Cx32 gap junction channels. Homotypic gap junction channels of Cx32N175H exhibited minimum gap junction conductance, which was significantly different from that of the wild-type Cx32 channels (**P<0.001); no significant difference was observed compared with the non-transfected N2A cells (control).
Cx32N175H could be rescued by Cx26 mutants
The results of Cx32N175H and our previous data on N175Y (Nakagawa et al., 2011) indicated that the mutations of the key hydrogen-bond-forming residue (Cx32N175H) and the corresponding residues from the non-compatible group of Cxs (H or Y) resulted in a substantial loss (4/6) or a total loss (6/6) of the hydrogen bonds at the E2–E2 docking interface; and in both cases the gap junction function was also lost. To explore the possibility of rescuing the function of the gap junction channel in these Cx32 mutants (N175H and N175Y), we designed two structurally complementary Cx26 mutants, K168V and K168A, and paired the Cx32 mutant hemichannels with these Cx26 mutant hemichannels. We reasoned that reducing the size and removing the positive charge of K168 might be necessary in order to spatially and electrically accommodate N175H/Y, and hopefully to restore the lost hydrogen bonds. The models of structural homology of these heterotypic mutant-docking pairs predicted that four inter E2–E2 hydrogen bonds were able to form in Cx32N175H/Cx26K168V (or K168A) (Fig. 3A); and three inter E2–E2 hydrogen bonds exist in Cx32N175Y/Cx26K168V (or K168A) (Fig. 3B). Our experimental data demonstrated that Cx32N175H could be rescued by either Cx26-K168V or K168A in terms of the formation of co-localized gap junction plaque-like structures at cell–cell junctions, and the formation of functional gap junction channels (Fig. 3). However, the same Cx26 mutants were unable to rescue Cx32N175Y by forming morphological and functional gap junction channels (Fig. 3). Apparently the number of the inter E2–E2 hydrogen bonds seemed crucial in deciding the functional status of the gap junction channel; and three hydrogen bonds in each docked E2–E2 pair appeared to be insufficient for proper docking and the formation of functional gap junction channels.

How many hydrogen bonds are required?
From the above results, it appeared that the number of the hydrogen bonds at the E2–E2 docking interface is crucial for the formation of morphological and functional Cx32/Cx26 heterotypic gap junction channels. With all the mutants in hand, we systematically examined a number of homotypic and heterotypic docking pairs between the mutant and wild-type Cx26 and Cx32. In order to obtain the predicted number of the hydrogen bonds and the functional status of the gap junction channel objectively, the homology modeling and experimental tests of the channel function were performed in parallel and double blind to each other. Fig. 4 summarizes all the docking combinations that were studied. The predicted number of hydrogen bonds between one pair of inter-docked E2–E2 and the functional status for each homotypic or heterotypic docking combination are presented (Fig. 4A). Furthermore, we plotted the Gf for all 20 docking combinations that were studied and ranked them according to the number of the hydrogen bonds per E2–E2 pair from zero to six (Fig. 4B). It is clear that among the combinations of homo- and heterotypic Cx26/Cx32 gap junction channels that were tested, a minimum of four hydrogen bonds per pair of inter-docked E2–E2 are required for the formation of a functional gap junction channel. These four hydrogen bonds could be either in a skewed (3+1) or in an even (2+2) distribution at the E2–E2 docking interface.

Our homology model predicted that the mutations of Cx26 at 168th position, K168A and K168V, reduced the number of hydrogen bonds at each E2–E2 docking interface from six to four for both of these mutant homotypic channels (supplementary material Fig. S1). The cellular localization of these Cx26 mutants appeared to be similar to those observed for wild-type Cx26, which could readily form gap junction plaques at cell–cell junctions (supplementary material Fig. S1). Furthermore, the levels of their cellular coupling conductance were also not different from those of Cx26 (Fig. 4B, bar C, D, A), indicating that this K168 or the equivalent residue is less important in docking and in the formation of functional gap junction channels probably because mutations at this position cause less impact on the number of hydrogen bonds formed at the E2–E2 interfaces.

Fig. 3. The mutant Cx32N175H, but not Cx32N175Y, could be rescued by pairing with designed Cx26 mutants. (A) Homology models for these mutant pairs (left panels) indicated that four hydrogen bonds were formed at the heterotypic E2–E2 interface in each case. When a GFP-tagged Cx32N175H-expressing cell was paired with an RFP-tagged Cx26 K168V (or K168A)-expressing cells, co-localization of Cx32N175H and Cx26K168V (or K168A) was identified at the cell–cell junctions. (B) By contrast, Cx32N175Y could only form three hydrogen bonds at the E2–E2 interfaces with Cx26 K168V and K168A; and no co-localized yellow gap junction plaques were evident in the heterotypic cell pairs. Scale bars: 10 μm. (C) Cx32N175Y could not form functional heterotypic gap junction channels with either Cx26 K168V or K168A, but the function of the N175H gap junction could be rescued by both Cx26 mutants. *P<0.05; **P<0.01; ***P<0.001.
were also included for comparison purposes. Some data from our previous study (Nakagawa et al., 2011) were presented; the alphabetical labels correspond to the bars shown in panel B.

**Fig. 4.** Proper docking and formation of functional gap junction channels from Cx32 and Cx26 require four or more hydrogen bonds at each E2–E2 docking interface. (A) All the Cx32/Cx26 docking combinations that were tested were summarized in Fig. 4A. The predicted number of hydrogen bonds at a pair of E2–E2 docking interfaces and the functional status of the gap junction channel (‘+’ means functional and ‘−’ means non-functional) are presented; the alphabetical labels correspond to the bars shown in panel B. (B) Gj for all the docking pairs examined was plotted against the number of the hydrogen bonds at the E2–E2 docking interface. The minimum number of the hydrogen bonds at one E2–E2 docking interface required to form a functional gap junction is four (or 4 × 6 = 24 hydrogen bonds for one gap junction channel). Some data from our previous study (Nakagawa et al., 2011) were also included for comparison purposes only.

### CMTX-linked Cx32 mutant N175D could be rescued by Cx26 mutant D179N

Interestingly, a mutation at the key hydrogen-bond-forming residue of Cx32 (N175D) was reported to be associated with CMTX disease (Silander et al., 1998). Under homotypic configuration, this mutant rarely formed putative gap junction plaques at cell–cell junctions (Fig. 5A) and was also unable to form functional homotypic gap junction channels (Fig. 5C). This was probably owing to the substantial loss of E2–E2 hydrogen bonds; according to our structure model only two inter E2–E2 hydrogen bonds were left (Fig. 5A). Coincidently, a structurally complementary Cx26 mutant D179N was found to be linked to non-syndromic hearing loss (Primignani et al., 2003). These two disease-linked mutants, Cx32N175D and Cx26D179N, can be considered as structure-reciprocal mutations between the two important hydrogen-bond-forming residues at the E2–E2 docking interface. The heterotypic gap junction channel between the Cx32N175D and Cx26D179N hemichannels was predicted to have five hydrogen bonds at each E2–E2 docking interface (Fig. 5B). Our experimental results demonstrated that the co-localized gap junction plaque-like structures of the heterotypic Cx32N175D/Cx26D179N channels were identifiable at cell–cell junctions (Fig. 5B); and the coupling level of the functional gap junction was also restored to the same level as that of wild-type Cx32/Cx26 heterotypic gap junction channels (Fig. 5C). In addition, owing to the differential charge selectivity for Cx32 and Cx26 hemichannels, the wild-type Cx32/Cx26 heterotypic channel exerted a characteristic asymmetric rectifying current (Suchyna et al., 1999). In a similar manner, Cx32N175D/Cx26D179N gap junction channels also showed rectified current (data not shown). Our data indicated that when docked with another disease-linked mutant, this CMTX-associated Cx32 mutant could be rescued in both the function of the heterotypic gap junction channel and the rectifying properties. These results also implicated that it is possible to rescue the function of certain disease-linked loss-of-function mutants through rationally designed connexin variants.

It is also noted that the rescue of the Cx32N175H and N175D gap-junction-channel function was also fully and partially achieved, respectively, by docking the two mutant-expressing cells with wild-type Cx26-expressing cells (Fig. 4, bars M and R, respectively). The homology modeling predicted that the number of the hydrogen bonds increased from two to four at each E2–E2 interface in both cases, which also met the minimum hydrogen bond requirement for the formation of functional gap junction channels (supplementary material Fig. S2).

### Discussion

In multi-cellular organisms including animals and human, gap junctions are evolved to mediate intercellular communication electrically and metabolically, and coordinate synchronized physiological activities in various tissues and organs. Different tissues and cell types often express different connexin isoforms, and in some cases one cell expresses multiple connexins. Thus, gap junctions within the same type of tissue cells and between different cell types can be quite complex, and numerous types of homotypic and/or heterotypic gap junction channels could occur. In addition, a lot of mutations from various Cxs have been found to associate with several inherited or sporadic human diseases. These Cx mutations can be autosomal dominant, recessive or somatic in nature, which give rise to even higher complexities, considering that a mutant Cx hemichannel could heterotypically interact with wild-type Cx hemichannels or a different mutant hemichannel. How hemichannels from one cell meet and interact with compatible hemichannels from another cell to form functional gap junction channels, i.e. the docking process, was not clear. However, this is fundamental for the intercellular communication through gap junctions in physiological and pathological conditions.

Gap junctions are unique intercellular channels spanning two layers of plasma membrane of the neighboring cells, which makes the crystallization of the intact gap junction channels extremely difficult. The first crystal structure of the gap junction channel was determined by Yeager and colleagues from truncated Cx43 at a resolution of 7.5 Å in the membrane plane (Unger et al., 1999), which depicted the Cx43 gap junction channel pore and revealed the alignment of the four transmembrane domains of each Cx subunit. However, the spatial resolution was very limited and unable to provide the structural details at the docking interface. Without the structural basis, some success on delineating the molecular docking mechanisms was obtained from the ‘blind’ testing of the function of gap junction channels in combination with site-directed mutagenesis, extracellular domain swapping and peptide assays derived from E1–E2 segments. These studies were able to identify the E2 domain and even several key residues...
Advantage of the 3.5 Å crystal structure of the Cx26 gap junction experiments and homology structural modeling. We took hence, Cx32 and Cx26 are ideal partners for both docking hemichannels exert inverse charge preference for permeate ions. Voltage impulses (Suchyna et al., 1999), because the two opposing asymmetric voltage gating properties, especially under higher Cx32 gap junction channels characteristically exhibit rectified and compatibility has been well established, and the heterotypic Cx26/Cx32 channels. The combination of structural insights especially in the gap junction docking interface (Foote et al., 1998; Perkins et al., 1998; Unger et al., 1999).

The recently published high-resolution crystal structure of the Cx26 gap junction channel (Maeda et al., 2009) provided a detailed structure of the docking interface, and revealed a total of 60 hydrogen bonds at all the E1–E1 and E2–E2 docking interfaces in one gap junction channel. Both Cx26 and Cx32 are important β group Cxs, which share the highest sequence homology between any two of the Cx family members. Their heterotypic docking compatibility has been well established, and the heterotypic Cx26/Cx32 gap junction channels characteristically exhibit rectified and asymmetric voltage gating properties, especially under higher voltage impulses (Suchyna et al., 1999), because the two opposing hemichannels exert inverse charge preference for permeate ions. Hence, Cx32 and Cx26 are ideal partners for both docking experiments and homology structural modeling. We took advantage of the 3.5 Å crystal structure of the Cx26 gap junction channel, and created homology models for mutant and wild-type Cx32 and heterotypic Cx26/Cx32 channels. The combination of structure modeling and morphological and/or functional examinations substantially advanced our knowledge on the docking process of the Cx26/Cx32 channels.

The crystal structure of the Cx26 gap junction channel and homology modeling suggested that the amino acid residues Cx32N175 and Cx26K168 (or their equivalent residues Cx26N176 and Cx32K167, respectively) in the E2 domains are essential for the formation of hydrogen bonds at the E2–E2 docking interface. Interestingly, based on E2 sequence alignment, and an assumption that at the docking interface the paired E2–E2 interact as anti-parallel β-sheets, the functional tests with paired Xenopus oocytes also pointed out the importance of these two amino acid residues for the function of the heterotypic channel (Nicholson and colleagues, unpublished observation) (for a review, see Harris, 2001). We previously described that the hydrogen bonds at the E2–E2 docking interface are important for the docking and formation of functional heterotypic Cx26/Cx32 channels (Maeda et al., 2009; Nakagawa et al., 2011). In the present study, we demonstrated for the first time that morphological and functional gap junction formation require a minimum of 24 (4 x 6) hydrogen bonds at six E2–E2 interfaces between two hemichannels of Cx26 and/or Cx32. More interestingly, the CMTX-linked Cx32 mutant N175D failed to form morphological and functional gap junction channels; however, this can be rescued by a designed docking partner to establish four or more hydrogen bonds at each E2–E2 docking interface. Coincidently, one ‘nature designed’ non-syndromic hearing loss mutant Cx26D179N – a structurally complementary mutant to Cx32N175D – was able to rescue the function of the gap junction channel of Cx32N175D by re-establishing a higher number of hydrogen bonds (five) compared with that of the threshold (four). Hence, our findings not only suggest a docking mechanism for these two β-group connexins, but also provide a better understanding of the molecular mechanisms involved in a disease-linked Cx mutant in humans and offer a potential rescue strategy.

Our homology models indicate that there are six hydrogen bonds at each E2–E2 docking interface of heterotypic Cx32/Cx26, homotypic Cx26 and homotypic Cx32 channels. They are clustered around two asparagines (e.g. Cx32N175 and Cx26N176 for heterotypic or the same asparagines for both homotypic channels). Each asparagine (Asp, N) makes three inter E2–E2 hydrogen bonds. Though slightly different for the heterotypic channel, the three hydrogen bonds on one Asp show rotational symmetry to the other three hydrogen bonds on the other docked Asp, which we defined as 3+3 in our figures. Reducing 1/3 (where the solidus represents one out of three) or 2/3 hydrogen bonds on one of the Asps did not impair the formation of hydrogen bonds at the E2–E2 docking interface. Interestingly, based on E2 sequence alignment, amino acid residues for the function of the heterotypic channel (Nicholson and colleagues, unpublished observation) (for a review, see Harris, 2001). We previously described that the hydrogen bonds at the E2–E2 docking interface are important for the docking and formation of functional heterotypic Cx26/Cx32 channels (Maeda et al., 2009; Nakagawa et al., 2011). In the present study, we demonstrated for the first time that morphological and functional gap junction formation require a minimum of 24 (4 x 6) hydrogen bonds at six E2–E2 interfaces between two hemichannels of Cx26 and/or Cx32. More interestingly, the CMTX-linked Cx32 mutant N175D failed to form morphological and functional gap junction channels; however, this can be rescued by a designed docking partner to establish four or more hydrogen bonds at each E2–E2 docking interface. Coincidently, one ‘nature designed’ non-syndromic hearing loss mutant Cx26D179N – a structurally complementary mutant to Cx32N175D – was able to rescue the function of the gap junction channel of Cx32N175D by re-establishing a higher number of hydrogen bonds (five) compared with that of the threshold (four). Hence, our findings not only suggest a docking mechanism for these two β-group connexins, but also provide a better understanding of the molecular mechanisms involved in a disease-linked Cx mutant in humans and offer a potential rescue strategy.

Our homology models indicate that there are six hydrogen bonds at each E2–E2 docking interface of heterotypic Cx32/Cx26, homotypic Cx26 and homotypic Cx32 channels. They are clustered around two asparagines (e.g. Cx32N175 and Cx26N176 for heterotypic or the same asparagines for both homotypic channels). Each asparagine (Asp, N) makes three inter E2–E2 hydrogen bonds. Though slightly different for the heterotypic channel, the three hydrogen bonds on one Asp show rotational symmetry to the other three hydrogen bonds on the other docked Asp, which we defined as 3+3 in our figures. Reducing 1/3 (where the solidus represents one out of three) or 2/3 hydrogen bonds on one of the Asps did not impair the formation of
functional gap junction channels (see Fig. 4 for 3+2 and 3+1 combinations). In a similar manner, functional gap junction channels were observed when reducing 1/3 hydrogen bonds on both Asps (2+2). These data indicate that in Cx32/Cx26 and/or their homotypic channels, the inter E2–E2 hydrogen bonds required to form functional channels are redundant; eliminating two out of six did not impair gap junction formation. However, hemichannels with combinations that eliminate all three hydrogen bonds on one (3+0), or 2/3 (1+1) and 3/3 (0+0) on both asparagines were unable to form functional gap junction channels, indicating the importance of these hydrogen bonds in the formation of gap junction channels. Interestingly, once the number of inter E2–E2 hydrogen bonds reaches four, any further increase in the number, e.g. to five or six, did not appreciably increase the Gj. In fact, the variations in Gj in the cell pairs with six inter E2–E2 hydrogen bonds appear to be more than the differences in the averaged Gjs between all the cell pairs with four and five, four and six, or five and six inter E2–E2 hydrogen bonds (see Fig. 4B). In other words, after reaching the ‘threshold’ for docking, a further increase in the number of the inter E2–E2 hydrogen bonds does not always increase the Gj, which depends on multiple factors, including the single-channel conductance and the probability of the channel being in the open state, and the abundance of gap junction channels at the cell–cell junction. From the sequence alignment, the key hydrogen-bond-forming residues of other members of Group 1 connexin are virtually identical to those of Cx26 or Cx32, and are likely to have a similar role in the docking process. However, at the corresponding positions of Group 2 connexins, the amino acid residues are quite different. If we could assume that the Group 2 connexin channels took the same or similar structure as that of Cx26 channel, then they would be unlikely to use hydrogen bonds as the main docking mechanism because the predicted number of inter E2–E2 hydrogen bonds would not be sufficient to reach the threshold (four per pair of E2–E2 hydrogen bonds), which was identified in the present study. The intermolecular interactions that are crucial for the docking of the Group 2 connexins remain to be identified.

In current literature, limited structural information is available on the docking and formation of functional gap junction channels. This study focused on the initial docking (or binding) recognition of the two hemichannels. The ‘opening’ of the whole gap junction channel is likely to be a subsequent process. We believe that cell-surface adhesion proteins are involved in bringing the two cell membranes into close apposition forming the ‘gap’ morphology (Harris, 2001), which allows cell-surface hemichannels to interact at the E2–E2 interfaces; for the Cx26 and/or Cx32 hemichannels, the inter E2–E2 hydrogen bonds might start to form. When sufficient E2–E2 hydrogen bonds (≥4 per pair or ≥24 per gap junction channel) had been formed, the two hemichannels would be docked properly and ready to ‘open’ as a functional gap junction channel. The open transition probably requires further conformational change at the E1 domains, part of which could be the previously described ‘loop-gating’ process (Kwon et al., 2012). Our earlier structural studies (Maeda et al., 2009; Nakagawa et al., 2011) indicated that the inter E1–E1 hydrogen bonds in Cx26 and/or Cx32 channels are also formed and might help to stabilize the gap junction channel, but they are unlikely to serve for docking recognition because the hydrogen-bond-forming residues are almost identical for both compatible and non-compatible groups of Cxs. These E1–E1 hydrogen bonds at the E1 docking interface and the intra-hemichannel E1–E1 interactions are more likely to form a tight barrel structure, sealing the aqueous channel pore from the extracellular space. The intra-connexin disulfide bonds between highly conserved cysteines might also be involved in stabilizing the channel pore conformation between the two docked hemichannels (Foote et al., 1998).

In summary, our study provided experimental evidence on the importance of the hydrogen bonds at the E2 docking interface for the formation of gap junction channels in a group of Cxs. Mutations on the crucial residue Cx32 N175 are predicted to lose most of the hydrogen bonds at the E2 docking interface, and indeed the functional homotypic gap junctions were not formed. Some of these Cx32 mutants could be rescued by docking to a designed mutant or wild-type Cx26, as long as the number of hydrogen bonds is equal to or higher than the threshold (four hydrogen bonds) at each E2–E2 docking interface. Such a rescuing strategy was further demonstrated in the functional rescuing of the CMTX-linked Cx32 mutant N175D. These findings suggest a general docking mechanism for a group of compatible connexins (Fig. 1) and offer potential rescue strategies for the disease linked Cx mutants in humans.

Materials and Methods

Sequence alignments

We performed multiple sequence alignments for homology modeling and analysis using the program ClustalW2 from the EBI package (Larkin et al., 2007). We selected Cx26, Cx30, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45, Cx46 and Cx50, which have been studied as homotypic and heterotypic connexin channels according to literature. Sequence identities of these connexins against Cx26 were >47%, and the lengths of the E2 domains are generally the same among the examined connexins. Consequently, Cx26 is an appropriate structural template for homology modeling. On the basis of their E2 sequences, these connexins were separated into two groups according to their heterotypic compatibility. The five connexins defined as group 1 are: Cx26, Cx30, Cx32, Cx46 and Cx50; the five connexins defined as group 2 are: Cx30.3, Cx37, Cx40, Cx43 and Cx45.

The construction of homology models for gap junction channels

The structure of the Cx26 gap junction channel was previously determined at a resolution of 3.5 Å (Protein Data Bank code 2ZW3) (Maeda et al., 2009; Suga et al., 2009). The homology models were created using the Coot program (Emsley and Cowtan, 2004). To generate Cx32 models, the amino-acid residues of a Cx26 hemichannel were replaced with the equivalent residues of Cx32 on the basis of the sequence alignments. To obtain a heterotypic or homotypic gap junction channel from different docking combinations between mutant and wild-type Cx32 and Cx26 hemichannels, one hemichannel was merged with the opposing hemichannel using the program PDBSET from the CCP4 package (Laskowski et al., 1993). Eventually, the molecular structures were refined by energy minimization without any structure-factor terms, by using the programs in CNS (Brünger, 2007). All molecular representations were created using PyMol (DeLano, 2006). The programs PROCHECK and SUPERPOSE of CCP4 were used to evaluate the quality and stereochemistry of the predicted models of heterotypic and homotypic channels (Krissinel and Henrick, 2004; Laskowski et al., 1993).

Non-covalent interaction analysis

The program CONTACT from the CCP4 package was applied to analyze the interactions between the two hemichannels of Cx32 and/or Cx26 (CCP4, 1994), with the following threshold values: O–N ionic bonds, 3.4 Å; O–O hydrogen bonds, 3.4 Å; O–N hydrogen bonds, 3.5 Å; N–N hydrogen bonds, 3.5 Å; S–O hydrogen bonds, 3.7 Å; and S–N hydrogen bonds, 3.7 Å.

Engineering Cx32 and Cx26 mutant cDNA constructs

The methods for generating wild-type and mutant Cx32–GFP and Cx26–RFP were described previously (Nakagawa et al., 2011). Cx32 N175H–GFP was generated with the following primers: forward 5′-CATTCCCTGCCCCCATACAGTGGACTGC-3′ and reverse, 5′-GACGATCCACGTGATGGGGGCAAGGGGTAG-3′. Cx32 N175D–GFP was generated with the following primers: forward 5′-TACCCCTGCCCCGACACAGTGGACTGC-3′ and reverse, 5′-GCAGTCCACTGTGTC-3′. Cx26-RFP was used as template to generate Cx26 mutant RFP constructs. Primers used to generate these mutants are: Cx26 K168A-RFP.
forward, 5'-CATCGACCGCTGCTGACGCTGTCGTCGGCGTGGC-3'; reverse, 5'-CAAGGCAGCGTGGCTGCTGCACTGTCGTCGGCGTGGC-3'; reverse, 5'-CGTCGGAGCGACCAAGCCCGCTGTCGTCGGCGTGGC-3'; reverse, 5'-ACAGGCGCTGCTGCACTGTCGTCGGCGTGGC-3'; reverse, 5'-GCTGCTGCACTGTCGTCGGCGTGGC-3'; reverse, 5'-AGACTTCTTGGTGGC-3'.

Cell culture and transient transfection

All reagents and culture media were from Life Technologies (Grand Island, New York, USA). HeLa and N2A (mouse neuroblastoma) cells were obtained from ATCC (American Type Culture Collection, Manassas, Virginia, USA). Cells were transfected with a GFP- or RFP-tagged mutant, or wild-type Cx32 and Cx26 by Lipofectamine 2000 (Life Technologies) as described previously (Gong et al., 2007). The expression of the fusion proteins was monitored using a fluorescence microscope.

Confocal imaging and patch-clamp electrophysiology

For homotypic channel experiments, 24 to 48 hours after transient transfection, the cells expressing Cx were ready for imaging and recording. In heterotypic channel configurations, 24 hours after transfection, the cells expressing GFP and RFP-tagged mutant or wild-type Cx32 and Cx26 by Lipofectamine 2000 (Life Technologies) as described previously (Gong et al., 2007). For confocal imaging, the cells grown on coverslips were fixed and then the nucleus was stained with Hoechst; after the coverslips were mounted to glass slides, images of the cells were captured on a Zeiss (Thorn-wood, NY, USA) LSM 510 META confocal microscope. Confocal microscopy and patch-clamp electrophysiology were carried out as described previously (Nakagawa et al., 2011). For confocal imaging, the cells grown on coverslips were fixed and then the nucleus was stained with Hoechst; after the coverslips were mounted to glass slides, images of the cells were captured on a Zeiss (Thorn-wood, NY, USA) LSM 510 META confocal microscope mounted on an inverted Axiovert 200 motorized microscope equipped with a 63×oil (1.4 numerical aperture) objective. Conductance of gap junction coupling between N2A cell pairs expressing GFP- or RFP-tagged Cxs was recorded using the dual whole cell patch-clamp technique. In homotypic configuration, isolated cell pairs with green or red fluorescent gap junction plaques at cell-cell contacts were selected for patch-clamp recording. In heterotypic compatibility testing, only cell pairs formed by one red fluorescent cell and one green fluorescent cell were selected for recording. Gj was calculated and presented as mean ± s.e. Online series-resistance compensation at 80% or offline series-resistance compensation were routinely applied to improve the accuracy of the measured Gj.

Author contributions

All authors participated in the design and writing of this paper. X.-Q.G. performed all the localization and double patch clamp experiments. S.N. and T.T. performed the homology modeling. D.B. is the principal investigator and obtained funding for the majority of this project.

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References


A gap junction docking mechanism revealed by functional rescue of a human disease-linked connexin mutant

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Supplementary Materials
Figure S1. Cx26 mutants retained majority of the E2-E2 HBs and were able to form GJ plaques at cell-cell junctions.

(A) A designed Cx26 compensatory mutant K168A-RFP trafficked to the cell-cell junction forming homotypic GJ plaques; according to homology modeling the Cx26K168A channel lost 2 out of 6 HBs at each E2-E2 interface. (B) When expressed in HeLa cells, another designed mutant Cx26K168V exerted similar cellular distribution and the ability to assemble into GJ plaques. (C) A structurally complementary mutant Cx26D179N was found to be linked to non-syndromic hearing loss, the residue replacement did not affect its E2-E2 HB number and its ability to form homotypic GJ plaques at cell-cell contact. Scale bars = 10µm.
Figure S2. When Cx32 and Cx26 mutants were docked with wild-type Cx26 and Cx32 respectively, the E2-E2 HBs were partially restored.

Upper row: when Cx26 mutants K168A and K168V were docked with wild-type Cx32, the number of the predicted HBs at each E2-E2 interface was 5, one more than their respective homotypic GJ channels. If Cx26D179N was docked with wild-type Cx32, the HBs at one E2-E2 interface remained the same (6 HBs). Lower row: when heterotypically paired with wild-type Cx26, Cx32 mutants, N175H and N175D, showed 4 HBs in each E2-E2 interface in comparison of 2 HBs for their homotypic GJ channels.