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Asymmetric configurations and N-terminal rearrangements in connexin26 gap junction channels

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Abstract

Gap junction channels are unique in that they possess multiple mechanisms for channel closure, several of which involve the N-terminus as a key component in gating, and possibly assembly. Here, we present electron crystallographic structures of a mutant human connexin26 (Cx26M34A) and an N-terminal deletion of this mutant (Cx26M34Adel2-7) at 6 Å and 10 Å resolution, respectively. The three-dimensional map of Cx26M34A was improved by data from 60° tilt images and revealed a breakdown of the hexagonal symmetry in a connexin hemichannel, particularly in the cytoplasmic domain regions at the ends of the transmembrane helices. The Cx26M34A structure contained an asymmetric density in the channel vestibule (“plug”) that was decreased in the Cx26M34Adel2-7 structure, indicating that the N-terminus significantly contributes to form this plug feature. Functional analysis of the Cx26M34A channels revealed that these channels are predominantly closed, with the residual electrical conductance showing normal voltage gating. N-terminal deletion mutants with and without the M34A mutation showed no electrical activity in paired *Xenopus* oocytes and significantly decreased dye permeability in HeLa cells. Comparing this closed structure with the recently published X-ray structure of wild-type

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Accession Numbers: The cryo-EM structures have been deposited in the Electron Microscopy Database (EMD, www.ebi.ac.uk/msd-srv/emsearch/index.html) with accession no. EMD-1748 for Cx26M34A and EMD-1749 for Cx26M34Adel2-7. The C-alpha coordinates and structure factors have been deposited in the Protein Data Bank with accession code 3IZ1 for Cx26M34A and 3IZ2 for Cx26M34Adel2-7.

Conflict of interest

The authors declare that they have no conflict of interest.

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Cx26, which is proposed to be in an open state, revealed a radial outward shift in the transmembrane helices in the closed state, presumably to accommodate the N-terminal plug occluding the pore. Because both the Cx26del2-7 and Cx26M34A^{del2-7} channels are closed, the N-terminus also appears to have a prominent role in stabilizing the open channel.

Keywords

Electron crystallography; gap junction channels; membrane protein structure; channel function; model fitting

Introduction

Investigations of gap junction channels have used independent but complementary experimental approaches to elucidate the gating mechanism of these intercellular conduits. Structural insights into the gating mechanism have been limited, however, by the lack of isolated channels in distinctly closed and open states. Previous electron crystallographic studies yielded a three-dimensional (3D)* structure of a C-terminal truncated connexin43 (Cx43) gap junction channel with each half containing 24 alpha helices arranged with a 6-fold symmetry.^{1, 2} Assignment of the helices to the primary sequence has been controversial, however, because functional mapping based on chemical pore blocks and structural models has led to varying conclusions.^{3–6} The recent 3.5Å crystal structure of the wild-type Cx26 (Cx26WT) provides a more detailed model that should serve as a basis to resolve these issues.⁶ We recently found that the 3D structure of a mutant human connexin26 (Cx26M34A) channels determined by cryo-electron crystallography showed an unexpected density within the vestibule of each hemichannel, which we called a “plug”.⁷ Our original experiments with this mutant showed significantly reduced dye coupling between HeLa cells transiently expressing Cx26M34A gap junctions.⁸ Projection maps calculated from images of two-dimensional (2D) crystals demonstrated a clear reduction in the plug density in the pore of an N-terminal deletion mutant of Cx26M34A, suggesting that the plug is composed, at least in part, of the N-terminus.⁹ Tracing of the Cα backbone in the recent X-ray crystal structure of Cx26WT showed that the N-terminus lies within the pore, against the pore wall.⁶ This “looping” of the N-terminus back into the pore is consistent with NMR structures of the N-terminal peptide of Cx2610 and Cx37, which contains a slightly longer sequence.¹¹

The role of the N-terminus in gap junction channel gating and trafficking has been further analyzed by mutagenesis studies. Within the connexin family, the N-terminus is the most conserved part of the three cytoplasmic domains. Deletions of up to 9 amino acids at the start of the Cx37 N-terminus do not interfere with trafficking to the plasma membrane in either *Xenopus* oocytes or mammalian cells, but N-terminal deletion eliminates hemichannel currents in single *Xenopus* oocyte experiments, indicating that this domain is required to maintain the open conformation of Cx37 hemichannels.¹² Mutagenesis studies of key residues in the Cx26 N-terminus suggest that this domain serves a critical role in channel gating as part of the transjunctional voltage sensor.^{13–15} Formation of the plug density by the N-termini could be an important feature of gap junction gating dynamics. Because connexins have multiple and complex gating sensitivities in response to voltage, pH, calcium, and various chemical modulators,¹⁶ however, this is probably not the only

* **Abbreviations:** Cx, connexin; Cx26M34A, Connexin26 site specific mutant Met34Ala; del2-7, N-terminus deletion of Cx26 missing amino acids 2-7; 3D, three-dimensional; 2D, two-dimensional; EM, electron microscopy; TM, transmembrane helix; WT, wild type; NCS, non-crystallographic symmetry; GFP, green fluorescent protein; PDB, Protein Data Bank

mechanism, and concerted movement of other parts of the structure is also likely to be involved in some gating responses.

In the present study, we present two electron crystallographic structures of the Cx26M34A mutant to determine the contribution of the N-terminus to the structure and function of gap junction channels. The first is a 6Å resolution structure of Cx26M34A channels, and the second is a 10Å map of an N-terminal deletion of Cx26M34A in which amino acids 2–7 were deleted (Cx26M34Adel2-7). We complemented these structural comparisons with electrical coupling and dye transfer experiments to assess the functionality of these mutants and to provide insight into the nature of this particular closed state of the channel. Finally, we fit the atomic model of the X-ray crystal structure of Cx26WT6 into a cryo-electron microscopy (EM) structure of Cx26M34A. No one structural technique or static view of a structure can fully explain complex gating movements of any channel and each crystallographic technique (2D EM versus 3D X-ray) delivers unique information about the channel structure and environment. The studies presented here indicate that connexin channels can adopt non-equivalent configurations with independent conformations of the six N-termini that cause an overall subunit rearrangement across the hexamer.

Results

Electron crystallographic structure of Cx26M34A at 6Å resolution

The structure of Cx26M34A was reconstructed from 179 images of 2D crystals (Table 1, Figs. S1a and S1b). The resolution of this new map was 6Å in the horizontal axis and 6.93Å in the vertical axis. Figure 1a shows a side view of the 3D structure of Cx26M34A obtained from 2D electron crystallographic methods. As in a previous study,⁷ we limit our description of the structure to a crystallographically averaged hemichannel from the middle lipid bilayer (Mem2) because flattening or surface tension effects minimally influence these hemichannels. The plug that we previously described in our 10Å structure⁷ is not 6-fold symmetric at this higher resolution, as might have been expected given the homomeric protein makeup of the hexamer. Rather, it has a 2-fold symmetric appearance, suggesting that the subunits within the Cx26M34A channel are not strictly 6-fold redundant. Two small protrusions on the top and four protrusions on the lateral face of the plug produce the asymmetric up-down configuration evident in the plug structure (Figs. 1b and 1b inset, white arrowheads). These protrusions are consistent with the plug comprising six N-termini,^{6,7,9} although we cannot rule out the possibility that parts of the flexible cytoplasmic loops contribute to the plug.

We fitted the coordinates of the Cx26 structure determined by X-ray crystallography (PDB code: 2zw3)⁶ to the cryo-EM density using a rigid body refinement.¹⁷ The first N-terminal 17 amino acid residues were excluded from this analysis because the N-termini adopt significantly different conformations in these two structures. The three crystallographically independent connexin subunits are labeled A (A', yellow), B (B', green), and C (C', cyan), and, for purposes of comparison, the four transmembrane helices (TM1-TM4) are marked as assigned in the 3D model of Cx26 determined by X-ray crystallography (Fig. 1b).⁶ The N-terminal loop is partially resolved as a small density from TM1 extending to a plug (Fig. 1b, red shaded densities). The cytoplasmic end of TM1 in subunit C rigidly interacts with TM2 in the adjacent subunit A' (Fig. 1c right panel, yellow arrow). This inter-subunit interaction is also observed between TM1 of subunit A and TM2 of subunit B (Fig. 1c left panel, green arrow), but is weaker between subunits B and C (Fig. 1c middle panel, cyan arrowhead). In subunit C, the cytoplasmic loop extending from TM3 curves extensively and interacts with the N-terminal end of TM1 (Fig. 1b, red arrows), a conformation that is not evident in subunits A and B. This interaction allows the second half of the cytoplasmic loop to regulate movement of the N-terminus within the pore. These asymmetric features are prominent

when comparing the original map with a 6-fold non-crystallographic symmetry (NCS) averaged map where the plug has a 6-fold blurred appearance and the cytoplasmic domains become featureless (Figs. 2a and 2b), and are still significant in the map at a higher contour threshold of 1.5σ (data not shown). An analogous situation occurs in the bacterial transporter protein, AcrB, in which each subunit of the homomeric trimer is structurally non-equivalent and functions independently of the others.¹⁸ Similarly, an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor, GluA2, was recently reported to comprise homo-tetramers with a symmetry mismatch between subdomains.¹⁹ The very flexible cytoplasmic domains of the gap junction channels are consistent with the connexin channel literature,²⁰ pointing to symmetry differences between the transmembrane/extracellular domains and the cytoplasmic-loop/C-terminal domain.

The N-terminal deletion mutant of Cx26 has a reduced plug density

To investigate the contribution of the N-terminal domain to the Cx26 structure and particularly to the plug density we observed, we determined the 3D structure of a partial N-terminal deletion mutant, Cx26M34Adel2-7, at 10Å horizontal resolution and 14.1Å vertical resolution (Table 1, Figs. S1c, S1d, and 3a). The crystal structure coordinates of a Cx26 monomer were fitted to each connexin subunit of the Cx26M34Adel2-7 cryo-EM density map, and the subunits of A^{del}, B^{del}, and C^{del} corresponded to subunits A, B, and C in Cx26M34A, respectively (Fig. 3b). The volume of the plug in the Cx26M34Adel2-7 pore was prominently decreased, consistent with the projection map,⁹ but a slim density was present in the pore center (Figs. 3a and 3b arrowhead). Because each Cx26M34Adel2-7 monomer lost only 6 residues at the N-terminal end, the rest of the N-terminus (~amino acids 8–23) had entered the pore, forming a partial plug. It is not possible to assign this density to any specific set of residues, however, because the 10Å resolution of this map is not sufficient to resolve the rest of the N-terminus. A similar situation is observed in the structures of plug-deletion mutants of SecY, a protein translocation channel, in which newly formed helical plugs seal the channel in the extracellular funnel.²¹

Functional analysis of Cx26 mutants

To correlate our structures with the functional state of the pore, the channel activity of Cx26 mutants was assessed electrophysiologically in paired *Xenopus* oocytes. Cx26 channels were expressed by injecting cRNA into paired *Xenopus* oocytes and junctional currents were monitored and compared to oligonucleotide-injected negative controls. Cx26M34A failed to induce measurable intercellular conductance in homotypic pairings (Fig. 4a). Heterotypic pairings of the mutant with Cx26WT revealed that this mutant formed functional gap junction channels, albeit with 90% reduced conductance. This is most easily explained if each hemichannel has an open probability (P_o) of ~0.1, which would mean that in homotypic mutant pairings, the open probability would be 0.01 (the product of the P_o of each hemichannel), and therefore below the limits of detection of the oocyte system. The electrical coupling of heterotypic pairings showed current decays in response to transjunctional voltage, similar to Cx26WT (Fig. 4b). Thus, Cx26M34A channels favor a closed state, but one that is distinct from that induced by transjunctional voltage, because the remaining open channels retain normal voltage gating.

Functional analysis of the N-terminal deletion mutants revealed no electrical activity for any 2 to 7-residue deletion mutant, with or without the M34A mutation (Fig. 4c). Dye coupling of these mutants was also significantly reduced, although not eliminated. Fusion constructs of Cx26WT, Cx26del2-7, and Cx26M34Adel2-7 with a green fluorescent protein-tetracycline tag (GFP-4C) at the C-terminus were also used in transiently transfected HeLa cells to monitor protein localization using GFP fluorescence. While all constructs formed gap junctions at cell apposition areas (Fig. 5d),⁹ less than 40% of the Alexa598-injected

cells were permeable in Cx26del2-7-GFP-4C and Cx26M34Adel2-7-GFP-4C (Figs. 5a and 5e) compared to 80% in Cx26WT-GFP-4C (Figs. 5a, 5b, and 5c). The reason for the greater reduction in the electrical coupling than in the dye coupling experiments is unclear, but might relate to the relative stability of the mutant channels in the oocyte and mammalian systems, recruitment of a very small population of endogenous Cx45 channels in the HeLa cells, or dye leakage between individual cell pairs. In both systems, however, partial deletion of the N-terminus dramatically reduced channel function, consistent with the report by Kyle *et al.* that Cx37 channels with various deletions of the N-terminus are non-functional.¹² Thus, while the N-terminus is involved in voltage sensing,^{13–15} it is apparently also required to maintain the channels in an open state, such that either a partial deletion or a point mutant that results in the formation of a pore “plug” reduces channel function. The N-terminus appears to be critical for stable assembly of connexin channels, as slightly larger deletions (e.g., 2–8 residues) reduced assembly of gap junction plaques, while deletions of 2 to 10 residues and 2 to 11 residues resulted in an almost complete loss of plaque formation and the accumulation of connexin protein in the cytoplasm (data not shown).

Fitting the X-ray structure of Cx26WT to the electron crystallographic structure of Cx26M34A

We compared the atomic model of Cx26WT channels as determined by X-ray crystallography,⁶ to our 6 Å Cx26M34A channel EM crystallographic structure. The first N-terminal 17 residues in the crystal structure were not included, as this region showed clear differences between the two structures, and we elected to focus on changes in the transmembrane segments. The transmembrane helices in all 6 subunits of Cx26M34A spread beyond the X-ray crystal structure through a radial tilt utilizing the extracellular docking surface as a fulcrum (Fig. 6, Supplementary movie). This was an unexpected observation, because the channels in the 3D crystals were surrounded by detergent micelles, whereas channels in the 2D crystals were embedded in the membrane. This expansion gave rise to a 45 Å-diameter pore at the cytoplasmic entrance, 5 Å larger than that in the X-ray crystal model structure (Supplementary movie). Our analysis suggests that connexin monomers are flexible enough to alter the channel diameters through a global subunit rearrangement.

Discussion

In this study, we present 3D structures of Cx26M34A at 6 Å resolution and Cx26M34Adel2-7 at 10 Å resolution, together with functional assays of channel activity. The findings based on these structures suggest that the N-termini within the channel pores facilitate global rearrangements and asymmetry in connexin subunits. Because the two structures we investigated contain a single amino acid mutation, these structural results require careful scrutiny to understand their significance with respect to a gating mechanism. In the absence of a 3D structure of Cx26WT or any WT connexin in the closed state, the relationship between the closed state induced by the M34A mutation and normal gated states of the channel is difficult to assess definitively. The conformational changes observed in these mutants, however, allow us to estimate the important roles of the M34 residue and the N-terminus, especially in promoting the flexibility of the connexin subunits across the hexamer and the stability of the open state of the channel.

Comparison of the EM and X-ray structures demonstrates that the subunits are radially expanded, with asymmetric configurations in membrane-embedded channels

The X-ray crystal structure of the Cx26WT channel was reported to be in an open conformation based on the crystallization conditions and the diameter of the pore in the 3.5 Å resolution map.⁶ In our “closed” Cx26M34A structure, the diameter of the pore constriction

(~15Å) formed at the TM1/E1 border was not very different from that of the “open” Cx26WT channel, whereas the cytoplasmic entrance of Cx26M34A was wider than that of Cx26WT. The greater width of the cytoplasmic entrance is caused by a radial expansion of the four helices, which is different from the rotational movement of subunits caused by Ca^{2+} .²² Ca^{2+} or aminosulfonate-modulated low pH gating is associated with a decrease in the pore diameter in atomic force microscopy images of force-dissected gap junctions (single hemichannel layers).^{23, 24} The atomic force microscopy topographs, however, were recorded from the extracellular side^{23, 24} and may reflect hemichannel gating in which the extracellular loops may adopt a different conformation from docked connexons.⁶

A plausible explanation for the closed state of Cx26M34A is the insertion of a plug density in the pore vestibule. This plug would be expected to result in a significant loss of electrical coupling (Fig. 4a) and decreased dye transfer.⁸ The plug density, which resides deeply in the vestibule and is asymmetric, appears to cause elastic spreading of the subunits laterally, but no significant change of the diameter deeper in the pore at the TM1/E1 border (Fig. 7). The up-down asymmetric configuration of the plug (Fig. 1b inset) implies that the bottom of the vestibule is too small to fit all six N-terminal helices, whereas the widening of the pore mouth appears to facilitate its placement. The cytoplasmic domains of connexin channels are labile and dynamic.²⁰ Widening the channel entrance would facilitate the placement of the N-termini as a unit, allowing the cytoplasmic domains to retain the flexibility that permits each subunit to assume an independent conformation (Fig. 7).

Alternatively, it is possible that expansion of the connexin subunits is caused by the differences in packing contacts and constraints in the 2D and 3D crystal forms. The 3D crystal form of Cx26WT contains longitudinal interactions between the cytoplasmic domains when the transmembrane domains are surrounded by detergent micelles.⁶ There are no visible lateral contacts in the electron density map,⁶ although there must be some for the 3D crystals to be stable. There is likely to be less lateral force compressing the subunits in the 3D crystals than in channels bounded by a lipid membrane, as in the 2D crystals or in the native cellular environment. Conversely, the channels in the 2D crystal interact in both the horizontal and vertical directions within lipid bilayers, but there are no end-to-end cytoplasmic domain contacts between hemichannels in apposing membranes. Based on these observations of crystal packing, the differences between the widths of the cytoplasmic entrance in the EM and X-ray structures are not likely due to crystal packing alone. It should be noted that a dehydration process was used to improve the resolution of the 3D crystals of Cx26WT.²⁵ It is unclear, however, whether protein dehydration alone could account for these subunit rearrangements.

Contribution of the M34 residue and the N-terminus to a unique 3D conformation and channel function

The point mutation of M34A is an analogue of Cx26M34T, a mutant that causes human hereditary deafness.²⁶ Despite some controversy about the dominant negative effect of the M34T mutation, genetic and functional data support the hypothesis that the M34 residue in Cx26 is essential for channel function.^{27–31} The aberrantly decreased permeability and conductivity of the M34A mutant derive from the introduced mutation⁸ (Fig. 4a). In the open state, the X-ray model of N-termini of Cx26 suggests that they are stabilized by a circular girdle of hydrogen bonds between D2 and T5, supported by the hydrophobic interactions between W3 (at the start of the N-terminal helix) and M34 side chains⁶ (Fig. 7). The M34 side chains allow the N-terminal helices to form a funnel structure that might be critical for maintaining an open state.¹² This hypothesis is supported by the observed loss of electrical function of Cx26M34A^{del2–7} channels, indicating the importance of an intact N-terminus for stabilizing the channel in the open state (Fig. 4c). This could also explain the aberrant activity of Cx26M34A (Fig. 4a) and Cx26M34T,^{27,30,31} where disruption of the

M34/W3 interaction may release the N-terminus from the pore wall and result in the formation of an occluding “plug” within the pore. Several other point mutations in the N-terminal loop, such as G12R, N14K, and S17F, are also associated with non-syndromic hereditary deafness.³² While we do not know the effects of these mutants on the N-terminus, each could easily disrupt the interaction of this domain with TM1 and the pore wall, resulting in channel occlusion by an N-terminal plug, or destabilization of the open state, or even loss of voltage sensitivity.³³

Biological relevance of non-equivalent subunit configurations to possible gating mechanisms

Six-fold non-crystallographic symmetry is generally applied to connexin channels at low or middle resolution to obtain better-defined channels or hemichannels.^{1·2·23·24·34–36} We also applied a 6-fold non-crystallographic symmetry to Cx26WT crystallized from channels expressed and purified with a baculovirus/Sf9 protein purification protocol, due to the limited initial resolution of these crystals. This increased the signal-to-noise ratio in each monomer, and facilitated the tracing of the protein backbone.⁶ It should be noted that NCS is widely used based on the assumption of homo-oligomeric redundancy. In this study, the 2-fold configuration of Cx26M34A channels demonstrated that connexin subunits act independently of each other. Previously, Oh *et al.* demonstrated that each connexin subunit in a Cx32 chimera hemichannel modulates its conformation individually in response to transjunctional voltage.³⁷ An analysis of the pH sensitivity of heteromeric junctional channels containing Cx32WT and a mutant of Cx32 with five arginine amino acids in the C-terminal domain replaced with asparagine residues demonstrated that the presence of even one Cx32WT monomer per hemichannel is sufficient to inhibit the acidification sensitivity of the five mutant monomers.³⁸ The findings of the present study demonstrate that this asymmetry may also have a role in homomeric channels, and could provide a mechanism by which multiple gating mechanisms are created in response to different stimuli.

Materials and Methods

2D crystallization of Cx26 channels and sample preparation for cryo-EM

All Cx26 constructs described in this paper were made using the human Cx26 sequence. Cx26 channels of Cx26M34A and Cx26M34A Δ 12–7 were purified⁸ and crystallized as described before.^{7·9} Briefly, purified histidine-tagged Cx26 proteins were mixed with decyl maltoside-solubilized 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) at a lipid-to-protein ratio of 0.8~1.2 (w/w). Dialysis was started against 10 mM MES, pH 5.8, 100 mM NaCl, 50 mM MgCl₂, 5 mM CaCl₂, 2 mM dithiothreitol, 100 μ M carbenoxolone (Sigma, St. Louis, MO), 0.005% NaN₃, and 1% glycerol. The concentration of NaCl was increased to 300 mM 3 days later. The dialysis solution was exchanged on the third day. The incubation temperature was kept at 20°C for the first day, 37°C for the next 4 days, and 20°C for the final day. The resulting 2D crystals were harvested from the crystallization button and mixed with a buffer containing a cryoprotectant (10% trehalose (Sigma, St. Louis, MO), 10 mM MES, pH 5.8, 300 mM NaCl, 50 mM MgCl₂, 5 mM CaCl₂, 2 mM dithiothreitol, 100 μ M carbenoxolone, 0.005% NaN₃, and 1% glycerol). The specimens were applied to carbon-coated molybdenum grids. The grids were blotted with filter paper and immediately frozen in liquid nitrogen using a plunge freezing device (KF-80, Reichert Jung, Austria).

Cryo-EM and image processing

All cryo images of Cx26 2D crystals were obtained at 4 kelvin using a JEOL JEM-3000SFF electron microscope at an accelerating voltage of 300 kV.³⁹ For Cx26M34A crystals, images of 0°, 20°, 45° and 60° tilt were recorded on Kodak SO-163 film at a magnification

of 40,000 $\times$ or 60,000 $\times$ with an electron dose of 25 electrons/ $\text{\AA}^2$. The Cx26M34Adel2-7 images of 0°, 20°, and 45° tilt were recorded at a magnification of 40,000 $\times$. Images that diffracted to better than 10 $\text{\AA}$ resolution using an optical diffractometer during an initial screening were then digitized with a SCAI scanner (Zeiss) at a step size of 7 μm per pixel. All images were processed with the MRC 2D crystal processing package⁴⁰ to correct lattice distortions. The contrast transfer function was corrected using square frequency filtering⁴¹ in combination with periodogram averaging.⁴² The statistics for the EM crystallographic structure determination of Cx26M34A and Cx26M34Adel2-7 crystals are shown in Table 1. The 6-fold NCS map of Cx26M34A was calculated by averaging three maps with rotations of 0°, 60°, and 120° around the c-axis. An inverse B-factor of -100 was applied to the 3D map of Cx26M34A to enhance the amplitudes of the high-resolution reflections. The 3D maps were visualized with the program Pymol (<http://pymol.sourceforge.net/>).

Preparation of cRNA and oocytes for microinjection

Oocytes were taken from ovarian lobe tissue, surgically removed from adult female *Xenopus laevis* after anesthetization in 0.2% tricaine chilled to 4°C to 6°C. The tissue was then separated into smaller segments with approximately 6 to 12 oocytes and incubated at 22°C for 1 hour in OR2 buffer (83 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 5 mM HEPES, pH 7.4 with NaOH) containing 1.5 mg/ml collagenase. After washing with OR2, stage V and VI oocytes were separated and manually defolliculated as necessary. They were then incubated at 17°C for 24 hours in half-strength L-15 medium (SIGMA), supplemented with 0.05 mg/ml gentamicin. Stage V/VI oocytes were injected with 23 to 46 nl of WT or mutant Cx26 cRNA along with 3 to 5 ng of XcCx38 antisense RNA for endogenous *Xenopus* Cx38 suppression.⁴³ Oocytes were then incubated for 1 or 2 days before analysis as single or paired entities.

Electrophysiological measurement

Oocytes were examined by voltage clamp experiments 18 to 48 hours after cRNA injection. Single oocyte voltage clamp recordings were taken using a two-electrode technique, while oocyte pairs were analyzed with dual electrode voltage clamps. GeneClamp 500B voltage clamps (Axon Instruments, Inc., Foster City, CA) were used in both single and paired oocyte arrangements. A 1 $\times$ L head stage was used for voltage recording and a 10 $\times$ MG head stage was used to pass current. The bath potential was actively clamped to 0 mV using a 100 $\times$ VG head stage. A horizontal puller (Flaming-Brown P-87, Sutter Instruments, Novato, CA) was used to make borosilicate glass electrodes used for passing current and recording voltage. Resistances for electrodes were between 0.5 and 3 megaohms when using the internal pipette solution (150 mM KCl, 10 mM EGTA, 10 mM HEPES at pH 7.2). The amplifiers were interfaced to a personal computer through a Digidata 1320 A/D converter. Data were acquired and analyzed using pClamp 8 software (Axon Instruments). Currents were acquired directly from the hard drive and filtered at 50 to 200 Hz. Attempts to induce current by injecting 5-fold the amount of RNA and waiting for periods of up to 72 hours did not lead to measurements significantly greater than those of control pairs.

Dye transfer assay

Parental HeLa cells were transiently transfected using Lipofectamine (Invitrogen, Carlsbad, CA) with human Cx26, Cx26del2-7, and Cx26M34Adel2-7 expressed as fusion proteins with GFP-4C in a pcDNA vector system. A solution of Alexa568 dye (2 mM in 200 mM KCl, Invitrogen) was injected into cells expressing each of the constructs as gap junctions (as first identified based on the GFP fluorescence at cell appositional areas). Parental HeLa cells were also injected in each experiment to serve as negative controls. Dye injections were performed on a BX50WI Olympus infrared differential interference contrast (DIC)/epifluorescent microscope (Olympus, Melville, NY) equipped with a 40X water immersion

objective. A fluorescein isothiocyanate filter set was used before injection to image the Cx-GFP-4C expression, and a rhodamine filter set was used after injection using to image the Alexa 568. Epifluorescent images of the Alexa568 dye were captured 5 minutes after each injection with a Nikon D300 digital camera and before and after images were superimposed. Dye transfer between adjacent cells was examined and statistics were then tabulated from each set of experiments.

Fitting the X-ray crystal structure of Cx26 to the cryo-EM density maps

Before fitting the X-ray crystal structure to the EM maps, we confirmed the magnification of the Cx26M34A structure by cryo-EM using aquaporin-4 crystal images as a calibration standard.⁴⁴ We processed 10 cryo images of Cx26M34A and aquaporin-4 2D crystals at a magnification of 40,000 $\times$. The differences in their magnification were less than 0.5%, and we concluded that magnification correction was not necessary for the cryo-EM structures in this study. The X-ray crystal structure of Cx26 containing amino acids 18 to 217 corresponding to a connexin monomer was initially manually fitted to each subunit in the cryo-EM structure on the program O,45 and then a more precise fitting of each Cx26 monomer as a rigid body was performed using a computational rigid body refinement with the SITUS program package.¹⁷ Although we compared helix assignments from the X-ray crystal structure with our EM structures, these assignments cannot be fully reconciled with functional studies.⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. 3D structure of Cx26M34A at 6 Å resolution

(a) Potential map of Cx26M34A, viewed parallel to the membrane plane, where a B-factor of -100 is applied. The three lipid bilayers are labeled Mem1, Mem2, and Mem3. The mesh contour level is 1.0σ (light blue). The scale bar is 40Å .

(b) Surface representation of the Cx26M34A map in Mem2 viewed normal to the membrane plane. The three crystallographically-independent connexin subunits are labeled A (A', yellow), B (B', green), and C (C', cyan), and the four transmembrane helices are labeled TM1 to TM4, as indicated in subunit C according to the coordinates of Cx26 (PDB code: 2zw3).6 White arrowheads highlight four protrusions from lower parts of the plug. In subunit C, the red arrows point to the turn in the cytoplasmic loop extending from TM3. Densities corresponding to the N-terminal loop are shaded red. (Inset) Magnified side view of the plug. Two protrusions on the top (two white arrowheads indicating upper parts of the plug) produce the up-down asymmetry.

(c) Interactions around the cytoplasmic end of TM1 for each subunit. Tight interactions of TM1-TM2 are observed between subunits B and A (left panel, green arrow) and subunits A' and C (right panel, yellow arrow), but TM1-TM2 appear to be disconnected between subunits C and B (middle panel, cyan arrowhead).

Fig. 2. Structure of Cx26M34A with 6-fold NCS applied

(a, b) Superposition of the original map (white surface) and the NCS map (yellow mesh) viewed normal (a) and parallel (b) to the membrane plane. In (b), the cytoplasmic end of subunit C is shown at higher magnification. Subunits are labeled A (A', yellow), B (B', green), and C (C', cyan). The cytoplasmic loop density in subunit C and C' (red arrows) and the plug (white arrowhead) exhibit different morphologies between the original and NCS maps. The cyan arrowhead indicates the density difference in the region of TM1-TM2 interactions.

Fig. 3. 3D structure of Cx26M34Adel2-7 at 10 Å resolution

(a) Potential map of Cx26M34Adel2-7 viewed parallel to the membrane plane. The three lipid bilayers are labeled as in Fig. 1a. The mesh contour level is 1.0σ (pink). The white arrowhead indicates the reduced plug density in the hemichannel embedded in Mem2. The scale bar is 40 Å.

(b) Surface representation of the Cx26M34Adel2-7 map viewed normal to the membrane plane. The connexin subunits are labeled A^{del} (A^{del}, yellow), B^{del} (B^{del}, green), and C^{del} (C^{del}, cyan) and TM1 to TM4 are labeled as in Fig. 1b. The white arrowhead points to the small density residing in the pore.

Fig. 4. Functional analyses of Cx26 channels

(a) Mean junctional conductances normalized to the average percentage of Cx26WT-Cx26WT pairings for several oocyte pairs. There is no significant conductance in Cx26M34A-Cx26M34A pairings. The heterotypic pairings of Cx26M34A-Cx26WT show a significant but reduced conductance.

(b) Representative whole-cell currents of Cx26WT-Cx26WT and Cx26M34A-Cx26WT subjected to a series of voltage steps ranging from -120 to $+60$ mV (30-mV increments shown above).

(c) Mean junctional conductances for the Cx26del2-7 mutants. There is no electrical coupling in any combination of Cx26 or Cx26del2-7 with or without the M34A mutation.

Fig. 5. Assessment of dye transfer of Alexa568 in neighboring HeLa cells in cells expressing gap junctions with human Cx26 N-terminal deletions and/or the M34A mutation

(a) Dye transfer is significantly decreased in HeLa cells transiently transfected with Cx26del2-7-GFP-4C and Cx26M34A del2-7-GFP-4C as compared to WT Cx26-GFP-4C. Alexa 568 was injected into the HeLa cells expressing gap junction plaques and dye transfer to apposing neighboring cells was measured after 5 minutes. Cx26WT served as a positive control, transferring dye in 4 of 5 injections, while no parental HeLa cells transferred dye in any of 8 injections. Cx26del2-7-GFP-4C and Cx26M34A del2-7-GFP-4C transferred dye to neighboring cells in 3 of 10 and 7 of 20 injections.

(b-e) An example of Cx26-GFP-4C cells showing extensive dye transfer is shown in (b and c), while (d and e) contain cells expressing Cx26del2-7-GFP-4C that had limited dye transfer (e, arrow). In (b and d), the GFP and Alexa 568 fluorescence are displayed as green and red, respectively, while (c and e) are grayscale versions of only the Alexa 568 fluorescence that are presented to better show the low levels of dye transfer (arrow in e). The scale bar is 10 μ m.

Fig. 6. Comparison between the X-ray and EM crystal structures of Cx26 reveals the global expansion of subunits across the hexamer in the EM crystal structure

(a) A model of the transmembrane and extracellular domains was created using a rigid body refinement of the fitted cryo-EM structure of Cx26M34A (orange). This model is superimposed on the X-ray crystal structure of Cx26 (red).⁶ The three crystallographically-independent subunits are labeled A (A'), B (B'), and C (C').

(b) Comparison of side views of the X-ray and EM models. The cytoplasmic ends of the Cx26M34A helices are tilted outwards from the pore compared to the Cx26WT helices. Orange arrows highlight domain movements in the Cx26M34A structure as compared to the Cx26WT model.

Fig. 7. Schematic of connexin movements

Secondary structural elements are depicted by green cylinders (α -helix) and green arrows (β -sheet). (Left) Representation of an open state based on the X-ray crystal structure. 6 Purple lines represent the second half of the cytoplasmic loop. The N-termini (red) are aligned annularly against the pore walls creating a conduit. M34 residues are marked on the TM1s. The black dotted line represents the unresolved cytoplasmic loop.

(Right) The conformational change as deduced from the cryo-EM structure of Cx26M34A indicates a global expansion of the subunits. The cytoplasmic end of TM1 can be modulated by the highly curved cytoplasmic loop (bold purple lines) at the entrance to the pore. The black dotted line represents the unresolved cytoplasmic loop. The cytoplasmic extensions related to the inter-subunit interactions are shaded magenta. The unresolved N-terminal loop is shown by the red dotted line. These movements may serve to facilitate the asymmetric positions of the six N-termini in the pore.

Table I

Summary of crystallographic data for the two cryo-EM 3D structures.

	Cx26M34A	Cx26M34Adel2-7
Space Group	p22 ₁ 2 ₁	p22 ₁ 2 ₁
Approximate tilt angle	Number of images	
0°	5	8
20°	28	50
45°	76	129
60°	70	0
Total	179	187
Cell parameters (Å)	a=112.4, b=111.2, c = 300.0 (assumed), $\gamma = 90^\circ$	a=113.4, b=112.2, c = 300.0 (assumed), $\gamma = 90^\circ$
Range of underfocus (Å)	4577 – 39,390	5443 – 22,135
Total number of observed reflections	36,007	19,943
Total number of unique reflections	3497	1534
Overall weighted phase residual (Used reflections are better than IQ 5.)	25.5°	26.3°
Resolution limit (Å)	6.0 (horizontal) 6.93 (vertical)	10.0 (horizontal) 14.1 (vertical)