Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule

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Connexin molecules form intercellular membrane channels facilitating electronic coupling and the passage of small molecules between adjoining cells. Connexin26 (Cx26) is the second smallest member of the gap junction protein family, and mutations in Cx26 cause certain hereditary human diseases such as skin disorders and hearing loss. Here, we report the electron crystallographic structure of a human Cx26 mutant (M34A). Although crystallization trials used hemichannel preparations, the density map revealed that two hemichannels redocked at their extracellular surfaces into full intercellular channels. These orthorhombic crystals contained two sets of symmetry-related intercellular channels within three lipid bilayers. The 3D map shows a prominent density in the pore of each hemichannel. This density contacts the innermost helices of the surrounding connexin subunits at the bottom of the vestibule. The density map suggests that physical blocking may play an important role that underlies gap junction channel regulation. Our structure allows us to suggest that the two docked hemichannels can be independent and may regulate their activity autonomously with a plug in the vestibule.

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Results

Two-Dimensional Crystallization of Connexin26 Complexes. hCx26M34A gap junction channels were expressed in and isolated from SF9 insect cells [see supporting information (SI) Fig. 7A]. Hemichannels (connexons) were isolated by affinity purification using a C-terminal hexa-histidine tag (SI Fig. 7B). Purified hemichannels were mixed with the lipid dioleoylphosphatidylcholine (DOPC) at a lipid-to-protein ratio of 1 (wt/wt). Reconstitution into lipid bilayers by dialysis produced 2D crystals >1 μm in diameter (SI Fig. 8A). Although the purified hemichannel is hexameric, the 2D arrays obtained by dialysis showed an orthorhombic crystal lattice (SI Fig. 8B).

We recorded images of the hCx26M34A crystals embedded in 0.05–1% tannic acid, 2–40% trehalose, or the combination of them. Computed diffraction patterns of one of the best 0° images showed reflections to a resolution of ~11 Å (SI Fig. 8C). After image processing to correct crystal distortions, the resolution improved to 7 Å (SI Fig. 8D). Image processing revealed that the crystals had h2221 symmetry and unit cell parameters of a = 112.4 Å, b = 111.2 Å, and γ = 90°.

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Abbreviations: Cx, connexin; Cx26M34A: connexin26 site-specific mutant Met34Aala; Data deposition: The cryoEM structure reported in this paper has been deposited in the Macromolecular Structure Database (Msd), www.ebi.ac.uk/msd-srv/emsearch/index.html (accession no. EMD-1341).

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Three-Dimensional Structure Determination and Organization of hCx26M34A Orthorhombic Crystals. To determine a 3D structure, we collected images of samples tilted up to 45° and combined them to produce a density map at a resolution of 10 Å in the membrane plane and 14.1 Å normal to the membrane plane (Fig. 1, SI Table 1, and SI Fig. 8E). A side view of the 3D map reveals that the crystals have a thickness of ~240 Å and contain three lipid bilayers (labeled Mem-1, Mem-2, and Mem-3 in Fig. 1). Remarkably, the map also shows that the hemichannels re-docked through their extracellular surfaces, forming complete gap junction channels (Fig. 1A and B). This is consistent with published results proposing extensive hydrophobic surfaces in

Fig. 1. Three-dimensional structure of Cx26 orthorhombic crystals. (A) Molecular packing of Cx26 in the 2D crystal. The gap junction channels are incorporated in three lipid bilayers (Mem-1–Mem-3) with 2₁ symmetry along Mem-2. (B) View of the Cx26 density map perpendicular to the membrane plane. The three membranes, indicated by gray bars, surround two extracellular gap regions. The map is contoured at 1.0σ (light blue) and 2.4σ (wheat color) above the mean density. The transmembrane α-helical ribbon model (6) is docked into the density for one of the hemichannels. The four helices are color-coded as in Fig. 2. Two helices D make contact with adjacent gap junction channels (red arrows). (Scale bar, 40 Å.) (C and D) Forty-angstrom-thick sections through the density map parallel to the membrane plane, showing protein embedded in membranes Mem-1 (identical to Mem-3) (C) and Mem-2 (D). Tail ends of two helices D are indicated by red arrows as in B. (Scale bars, 40 Å.)
the gap region (14). In bilayers Mem-1 and Mem-3, the hemichannels show poorer density than in Mem-2 (Fig. 1 C and D), presumably because of variability in the molecular packing because of the large lipid areas between individual hemichannels and/or the flexibility of the cytoplasmic domains of the connexin subunits. The cytoplasmic structures in Mem-1 and Mem-3 may also be deformed by their contact with the carbon film to which the crystals are adsorbed in the sample preparation procedure for cryoelectron microscopy. By contrast, the hemichannels in Mem-2 are protected from any forces such as the surface tension upon specimen drying and mechanical interactions with the carbon film. Therefore, the structural features of the hemichannels in Mem-2 should be the most accurate and, in particular, preserve the structure of the flexible cytoplasmic domains of the connexins. Thus, the following description of the gap junction structure is based on the hemichannels in Mem-2 unless noted otherwise.

Each connexon in a gap junction membrane channel contains four transmembrane segments, usually referred to as M1, M2, M3, and M4 (15). The combination of our x-y resolution of 10 Å and the well defined cytoplasmic and transmembrane structure made it possible to unambiguously dock the proposed ribbon model of the transmembrane domain (PDB accession no. 1TXH) with slight modifications (Fig. 2). A comparison of the transmembrane cylinder models for Cx43 (6) with our Cx26 structure (Fig. 3) show that the dimensions of the channel, the size of the pore constriction, and the positions of the helices are all essentially the same. It should be noted that Cx26 is part of the β-subgroup of the connexin family, whereas Cx43 is a member of the α-subgroup, yet the overall structure of these two connexin isoforms is very similar. Small shifts in the positions and tilts of these superimposed helices are more likely because of the different crystal forms used in the structure analysis for Cx43 and Cx26 (hexagonal versus orthorhombic) than differences between the isoforms.

There are three protrusions on the cytoplasmic surface for each four-helix bundle, A’, B’, C, and D (Figs. 2 and 4). The cytoplasmic extension of helix D makes contact with the one from the adjacent gap junction channel, stabilizing the crystal packing (red arrows in Fig. 1 B and D). Four-helix bundles are connected by a bridge-like density forming the largest of the three cytoplasmic protrusions (white arrows in Fig. 2; and see Fig. 4). This new density could
represent part of the cytoplasmic loop of Cx26, because this domain caps the top of helices B and C. The cytoplasmic loop has a larger number of amino acid residues than the N or C terminus, and thus should have a larger mass density. This implies that the connexin polypeptide boundary may be across adjacent four-helix bundles. However, flexible loops are often invisible in crystallographic maps. The two loops in the extracellular gap domain are not clearly separated (Fig. 2C) and are postulated to form a differently organized subdomain (16). At this time, the resolution is insufficient to assign these α-helical segments and the polypeptide boundary to specific sequences within the transmembrane domain.

**The Prominent Pore Density in the Vestibule.** The 3D map shows a density in the center of the pore (white arrowheads in Figs. 2 and 4). Because this density is observed in both projection maps of tannic acid- and trehalose-embedded specimens (data not shown), it is most likely that the plug is part of the connexin and not an artifact of the embedding agent. This plug density is clearly visible even in a map contoured at 2.4σ (wheat-colored contour in Fig. 2). The plug is located inside of the membrane layer and forms contacts with the surrounding channel wall, which, at the constricted part of the vestibule, is formed by the innermost helices C (Fig. 2B). This density strongly suggests that a plug physically blocks the channel within the membrane. Density for the plug is also observed in the pores of the hemichannels in membranes Mem-1 and Mem-3 (Fig. 1 B and C). Each hemichannel has its own plug, conferring on it the ability to gate its pore autonomously. It is possible that the transjunctional voltage sensor and the physical gate reside exclusively within a single hemichannel (7, 17).

The connection of the plug to the top of the channel wall is not perfectly resolved, presumably because of flexibility of the linker (Fig. 2 Insert). We applied a B-factor of −700 to the map to enhance the higher-resolution weak amplitude data. This map shows that the plug consists of six α-helix-like features and that it has four protrusions, implying connections from the plug (Fig. 5). It is not surprising that only four protrusions rather than six appear from the plug because 6-fold symmetry is lost in this orthorhombic crystal form. These features strongly suggest that the plug is formed by either the N- or C-terminal helices of Cx26.

**Discussion**

Key findings of this study are that hydrophobic interactions at the extracellular domains drive hemichannels to redock into dodecameric channels, the transmembrane domain structure is fairly conserved between α- and β-connexins and different crystal forms, and each hemichannel has its own plug in its vestibule. The crystal form shown here is unprecedented in the gap junction structure literature because it contains three membranes and two sets of symmetry-related intercellular channels. This could occur only because removal of the detergent from hemichannels causes the hydrophobic extracellular surfaces to be exposed. Rather than have these surfaces face an aqueous environment, hemichannels redocked into an intercellular channel. Comparison of the shape, size, and arrangements of the transmembrane helices in the hCx26M34A structure with the truncation mutant of Cx43 show that the architecture of the gap junction channel is conserved between these two isoforms. Without the resolution to unambiguously trace the primary sequence of the connection from the plug to the surrounding channel wall, we can only speculate that the plug arises from one of the termini, in particular, the N terminus.

**Possible Candidates for Components of the Plug.** Our Cx26 gap junction crystal structure shows that the channel vestibule is blocked by a physical obstruction we call the “plug.” Whether this structure reflects a functionally closed nonpermeant channel or an aberrant mutant channel remains to be determined. However, it is unlikely that a single amino acid change by itself would give rise to such a prominent feature. We have obtained several 0° projection micrographs of wild-type 2D crystals whose crystallinity is not as good as crystals of the hCx26M34A mutant. These 2D reconstructions have also revealed the density in the pore (data not shown). This last finding strongly implies that the physical channel closure may be regulated independently in each of the hemichannels and that this plug may make a channel impermeant to larger molecules such as those used in our previous dye-transfer studies (13).

The likely candidates for the plug and its connector are the N terminus, the cytoplasmic loop, and the C terminus of Cx26. The N terminus is the most probable candidate of the three. A wealth of electrophysiological studies has established that the transjunctional voltage sensor resides in the N terminus and that the residues sensing changes in the voltage field are located in the vestibule of the pore (7–9, 18). It has also been proposed that movement of the charges in the N terminus initiate channel gating (7, 19). This notion is supported by an NMR solution structure of an N-terminal peptide of Cx26 (20), although it has not yet been shown definitively that the N terminus forms the physical assembly. We propose that the plug, which is located within the pore region, and is therefore ideally located to detect...
the transjunctional voltage field, contains the N termini of the connexin subunits. This is different from voltage-sensitive K⁺ or Na⁺ channels, where the S4 domain is the voltage sensor but does not form an assembled plug (21). It should be noted that sequence analysis has shown that only N termini have very similar lengths and a highly conserved sequence, but both the cytoplasmic loops and the C termini show variable lengths and less sequence conservation (22). This sequence conservation of the N terminus might, in turn, result in conservation of the plug feature and closing mechanism within the connexin channel family.

The C terminus is the next most likely candidate for the plug. The Cx26 construct in this work has a hexa-histidine tag with a thrombin digestion recognition sequence linker that results in ≈30 aa residues assigned to the C-terminal tail and makes it slightly longer than the N-terminal tail (21 aa residues). It has also been proposed that the C terminus and the part of cytoplasmic loop is involved in pH regulation of gap junction gating, which is referred to as “particle-receptor” model (23), although there is no direct evidence that these domains interact within the vestibule. Whereas Cx26 channels do gate at low pH (24), this model has not been demonstrated for Cx26 channels because of the very short C-terminal tail. Another mechanism has been proposed for pH gating of Cx26 hemichannels (25, 26), although it has been reported that addition of tags to the Cx26 C terminus eliminates the ligand binding that promotes pH-induced channel closure (27, **). Furthermore, we found that connexins without the C-terminal tag never crystallized, and removal of the tag after crystallization destroyed the crystals (data not shown). It is more likely that the end of helix D corresponds to the hexa-histidine tag that serves to stabilize crystal formation rather than forming plug. The idea that the C terminus extends from helix D is consistent with the assignment of helix D as M4 as suggested (6, 28).

The last candidate for the plug would be the cytoplasmic loop of Cx26, however, the number of amino acids (≈35 residues) and requirement that this part of the sequence makes a loop cannot account for connecting densities from the plug (Fig. 5). Previously published work has suggested that the N terminus and cytoplasmic loop of Cx26 may interact directly (18). Considering that even the largest cytoplasmic protrusion could be too small to cover the length of the cytoplasmic loop (Figs. 2 and 4), it is possible for the N terminus and the cytoplasmic loop to cooperate with each other, resulting in forming the plug.

The Plug Creates a Blocked State of Cx26 Gap Junction Channel. Since the M34T substitution of hCx26 was reported to be the cause of nonsyndromic hearing loss (11), the functional role of the position 34 has been studied, and it has been suggested that M34T leads to a constriction of the channel pore (12). In our work, it is conceivable that the plug in the pore is locked into place by the hCx26M34A mutation because the physical obstruction is consistent with the decreased permeability of the hCx26M34A mutation (13). In this case, the mutation at the M34 site in M1 could affect the movement of the M1 helix in each connexin in each hemichannel, thus resulting in a change of the plug position that makes the channels nonfunctional or poorly functional.

It should be noted that not only have we used a permeability mutant for this study but also that we have used crystallization conditions (low pH, aminosulfonate buffer, carbamoxolone, and high Ca²⁺ and Mg²⁺) that generally promote a closed Cx26 channel. The remaining pore openings at the constriction site are ≈8 Å in diameter (Fig. 2B), indicating that these openings are too small for fully hydrated ions to pass (29). If our structure of the blocked state resembles a functionally closed state because of those factors in the crystallization condition, it allows us to suggest a plug gating mechanism. Specifically, the two interacting Cx26 hemichannels can gate their channel directly and autonomously with a plug (Fig. 6). In this model, a Cx26 gap junction is open only when both hemichannels release their plugs toward the cytoplasmic side. Once open, the pore can immediately conduct large molecules such as peptides with a molecular mass of up to 1.8 kDa (30), because the large pore size (≈15 Å

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Fig. 5. Stereo top view of the Cx26 density map to which a B-factor of −700 was applied. The B-factor was applied to enhance the amplitudes of the high-resolution reflections, revealing six α-helix-like features in the plug density and protrusions that probably reflect the loops connecting the plug to the surrounding channel wall. The four helices are color-coded A (cyan, A’), B (green, B’), C (yellow), and D (pink) as in Fig. 2.

Fig. 6. Hypothesized plug gating mechanism of gap junctions. Each hemichannel (green) can regulate its channel activity autonomously. The gap junction is open only when the plugs (red) in both hemichannels are displaced from the channel constriction formed by the innermost helices C (yellow) toward the cytoplasmic side. The flexible connections of the plug with the channel are shown as red dashed lines.
in the most constricted region) virtually does not change during the gating cycle. Given that our structure and others (15) have indicated that the cytoplasmic domains of connexin are very labile, the movement of the plug could also be regulated by interactions with other cytoplasmic connexin domains, which might account for the diversity of channel properties between different connexins.

In either case, the fact that each hemichannel has its own plug supports the argument that the relationships between two opposing hemichannels can be independent of one another. This notion well reconciles the observation of functional conductive posing hemichannels can be independent of one another. This supports the argument that the relationships between two open connexins.

Different concentration of tannic acid or trehalose did not affect the plug in the pore because all of the 0° projections obtained from any preparations showed the plug density as well (data not shown). After removal of excess liquid, the grid was blotted with filter paper and plunged into liquid nitrogen.

**Cryoelectron Microscopy and Structure Determination.** Frozen specimens were transferred into a JEM-3000F electron microscope (JEOL, Tokyo, Japan) operated at 300 kV and equipped with a field emission gun and a superfluid helium stage (34). The specimens were cooled to a temperature of 4K, and images of specimens tilted up to 45° were recorded on SO-163 film (Kodak, Rochester, NY) at a magnification of ×60,000 with an electron dose of 25 electrons per Å². The quality of images was checked by optical diffraction, and selected images were digitized with a SCAI scanner (Zeiss, Thornwood, NY) by using a step size of 7 μm. Lattice distortions and the contrast-transfer function were corrected with the MRC package (35–37). The final density map is based on 254 images that were combined to generate a merged phase and amplitude data set. The 3D density map was visualized with the program Pymol (http://pymol.sourceforge.net/).

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**Materials and Methods**

**Protein Purification and 2D Crystallization.** hCx26M34A hemichannels were purified as described (13), with the minor modification that the protein was eluted with 300 mM imidazole instead of 300 mM imidazole. Purified hCx26M34A hemichannels were not have any plug density in the pore (5, 6), further studies are necessary to verify whether the plug structure can be generalized to all connexins. Structures at higher resolution of the open and closed states will provide more structural detail and will be needed to fully understand the functional role of a plug and gating mechanisms in these widely expressed channels.
