Roles of Met-34, Cys-64, and Arg-75 in the Assembly of Human Connexin 26

IMPLICATION FOR KEY AMINO ACID RESIDUES FOR CHANNEL FORMATION AND FUNCTION*

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Connexins form a family of membrane proteins that assemble into communication channels and directly connect the cytoplasm of adjoining cells. Malfunctioning of connexin channels often cause disease, such as the mutations M34T and R75W in human connexin 26, which are associated with hereditary deafness. Another residue known to be essential for normal channel activity in the connexin is Cys-64. To obtain structural and functional insights of connexin 26, we studied the roles of these three residues by expressing mutant connexins in insect Sf9 and HeLa cells. The M34T and M34A mutants both formed gap junction plaques, but dye transfer assays showed that the M34A mutant had a significantly reduced permeability, suggesting that for proper channel function a side chain of adequate size is required at this position. We propose that Met-34 is located in the innermost helix of the channel, where it ensures a fully open channel structure via interactions with other transmembrane helices. Gap junction channels formed by the R75W and R75D mutants dissociated upon solubilization in dodecyl maltoside, whereas the R75A mutant remained hexameric. All gap junctions formed by Arg-75 mutants also showed only negligible activity in dye transfer experiments. These results suggest that residue Arg-75 plays a role in subunit interactions needed to retain a functional and stable connexin hexamer. The C64S mutant was suggested to be defective in oligomerization and/or protein folding even in the presence of wild-type connexin.

Gap junctions are assemblies of intercellular communication channels that mediate the direct transfer of metabolites, cytoplasmic ions, and molecules lower than about 1 kDa in size between neighboring cells (1). Gap junctions contribute to various biologically important processes such as cell growth, normal organ function, embryogenesis, and development (2). Whereas all connexin (Cx) subtypes share a common topology consisting of four transmembrane helices (M1–M4), two extracellular loops (E1 and E2), and cytoplasmic N and C termini (3–7), the main differences are found in their C-terminal tails. Six connexin subunits assemble into a hexameric hemichannel, named connexon, and the docking of two connexons from adjacent membranes produces a complete gap junction channel, resulting in a narrowing of the extracellular gap between adjacent lipid bilayers to about 40 Å (8). These characteristic structures have not yet been found in prokaryotes, indicating the necessity for more complex cell-cell communication networks in higher organisms.

First structural studies were performed on rat liver gap junctions, mostly composed of connexin 32 (Cx32) and connexin 26 (Cx26) (9, 10), which confirmed the model of gap junction channel with six membrane-spanning subunits forming the oligomeric channel through the plasma membrane (11, 12). Electron crystallography further demonstrated that a connexin channel is formed by two hemichannels docking to each other with a rotational displacement (13, 14). More recently, electron crystallography was used to study the three-dimensional structure of C-terminally truncated recombinant connexin 43 (Cx43) gap junction channels, and the 7.5-Å (21 Å in the vertical direction to the membrane surfaces) three-dimensional map revealed for the first time the arrangement of the α-helices in gap junctions (15). Despite our growing understanding of the structure of gap junction channels, an atomic model is still lacking. Knowledge of the gap junction channel in atomic detail is needed, however, for a complete understanding of the molecular basis for the function of this important family of channel-forming membrane proteins.

Mutations in the Cx26 gene have been proven to be responsible for nonsyndromic neurosensory autosomal deafness. Cx26 is expressed in the cochlea of rat and human (17, 18), where it is thought to mediate the regulation of the potassium ion flux from sensory hair cells to the spiral ligament or the spiral limbus through supporting cells (19). The importance of Cx26 for cochlear functioning is elucidated with targeted ablation mice, suggesting that the apoptotic process is essential for the auditory function (20). The M34T mutation was first identified to cause the deafness and shown to have a dominant-negative effect by preventing functional channel coupling (18, 21, 22). However, the dominant-negative effect of the M34T mutation has been argued, because families carrying the M34T mutation homozygously or heterozygously exhibit recessive phenotypes (23–28), and the exact role of residue Met-34 in the context of the gap junction channel is still unclear. Whereas the

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† The abbreviations used are: Cx, connexin; DoDM, dodecyl maltoside; GFP, green fluorescent protein; WT, wild type; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ER, endoplasmic reticulum.

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R75W mutation in Cx26 was originally found in a family displaying both deafness and palmoplantar keratoderma and was shown to have a dominant-negative effect by paired Xenopus oocyte experiments (29), no report studying the effect of the mutation on gap junction assembly was published yet. Residue Arg-75 is located at the very beginning of the second transmembrane helix (M2). This residue as well as the sequence around it, is highly conserved among all connexin subtypes (29), indicating that this residue plays an important role in all gap junctions.

It is noteworthy that the motifs of extracellular cysteines are conserved in all the connexin subtypes with the sole exception of Cx31. Previous studies (30, 31) demonstrated that the cysteines might be involved in disulfide bonds, implying a role in stabilizing the characteristic fold of the extracellular domains. Moreover, a mutational analysis showed that substitution of any one of the six cysteines in Cx32 to serine resulted in loss of channel conductance in paired Xenopus oocyte experiments (32, 33). Based on studies using systematic mutations of conserved cysteines, a β-sheet structure was proposed for the extracellular loops (34).

In this report, we studied the roles of residues Met-34, Cys-64, and Arg-75 in human Cx26 by introducing mutations at each position, focusing on the effect on formation of connexons and gap junction plaques. Because it has been shown that connexins expressed in insect Sf9 cells form gap junction plaques in the insect cell membranes (35), we used this system to express mutant connexins. All mutants were also fused to GFP at the C terminus and transiently expressed in HeLa cells to investigate their trafficking to the plasma membrane and to characterize their channel activities (36). Our results suggest that the three residues studied here all play important but different roles in gap junction channels. Here we propose a model for the location of the molecular structure and functional roles of these key residues of Met-34, Cys-64, and Arg-75 based on our mutational studies of Cx26 combining with results from previous physiological, functional, and structural studies.

MATERIALS AND METHODS

Construction of WT and Mutant Cx26 Genes in Transfer Plasmids—For expression in Sf9 cells, human Cx26 cDNA (37) was ligated into the EcoRI/HindIII sites of the pVL1393 vector (Invitrogen). Point mutations were introduced using the following oligonucleotide primers: M34T, 5'-CTATTTTTCGATCGATTGCGATCCTCGTTGTGGCT-3'; C64S, 5'-CGAGGC-CCATAGCGGAGGAATGGGAAAATGTA-3'; R75W, 5'-CAGGGG-CCATAGCGGAGGAATGGGAAAATGTA-3'; M34A, 5'-TT- CATATTTTTCGATCGATTGCGATCCTCGTTGTGGCT-3'; M34G, 5'-TT- CATTATTTTTCGATCGATTGCGATCCTCGTTGTGGCT-3'; M34S, 5'-TT- CATTATTTTTCGATCGATTGCGATCCTCGTTGTGGCT-3'.

For expression in HeLa cells, the genes were introduced into pcDNA3.1(+) vector (Invitrogen) and the pBlueBac4.5 vector (Invitrogen). Tagged genes were cut with 5' and 3' EcoRI sites. A sequence encoding KTETSQVAPA, the epitope for the anti-rhodopsin monoclonal antibody 1D4, was attached at the C terminus of Cx26 to enable protein purification by nickel-affinity chromatography. For expression in HeLa cells, the genes were introduced into pcDNA3.1(+) vector (Invitrogen) and the pBlueBac4.5 vector (Invitrogen). Tagged genes were cut out from the pcDNA3.1(+) vector by EcoRI and BamHI treatment and inserted into pEGFP-N3 vectors (Clontech) using the EcoRI/BamHI sites.

Sf9 Cell Culture and Membrane Preparations—Recombinant viruses were prepared with linearized BaculoGold DNA (Phar mingen) or linearized Bac-N-Blue DNA (Invitrogen) following the protocols given by the manufacturers. After transfection, single viruses were isolated using the plaque assay. Sf9 cells were grown in 400-ml suspension cultures as described (38) using SF900II-SFM medium supplemented with 2% fetal bovine serum, 0.1% Pluronic F68, and antibiotic-antimycotic (Invitrogen). At a density of 1.5 × 106 cells/ml, Sf9 cells were infected with recombinant virus at a multiplicity of infection of 1–2 and cultured for 90 h. When wild-type and mutant viruses were co-transfected, wild-type:mutant virus ratios of 2:1 or 3:1 were used because the wild-type virus usually showed a lower expression level than those of the mutants (see "Results"). Cells were harvested, washed with PBS, frozen in liquid nitrogen, and stored at −80 °C until further use. Membranes were enriched in gap junctions using the alkaline extraction method (35). Membrane aliquots corresponding to 100-ml culture cells were frozen and stored at −80 °C. For EM analysis, 2 ml of membranes were applied to carbon-coated copper grids and negatively stained with 2% uranyl acetate.

Membrane Solubilization, Protein Purification, and Analytical Gel Filtration—Solubilized membranes were pelleted and sonicated in solubilization buffer (1 M NaCl, 10 mM HEPES, pH 7.5, 2% DDM, 0.005% NaN3) and incubated for 2 h at 4 °C. After centrifugation at 100,000 × g at 4 °C for 30 min using a Beckman TLA100.3 rotor, the supernatant was incubated with Ni-NTA beads (Qiagen) in 10 ml imidazole for 60 min. The beads were washed with washing buffer (1 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% DDM, 0.005% NaN3, 100 mM imidazole), and proteins were eluted with 250 mM imidazole. Protein concentrations were determined with the BCA kit (Pierce). For thrombin digestion, the purified proteins were treated with thrombin (0.1 units/300 μg of proteins) at room temperature overnight. The oligomeric state of connexins was determined by analytical gel filtration. 500 μl of protein solution was filtered through a Ultrafree-MC (Millipore) using a 0.22-μm filter and eluted in a Superose 6 HR10/30 column (Pharmacia-LKB Bio- sciences) in 500 mM NaCl, 10 mM HEPES, pH 8.0, 0.2% DDM, 10 mM DTT, 0.005% NaN3, at a flow rate of 0.4 ml/min. 1 ml fractions were collected and analyzed by SDS-PAGE using 10–20% gradient acrylamide gels, and the protein bands were visualized by silver staining.

Electron Microscopy—Negatively stained samples were examined with a JEOL 1010 electron microscope operating by magnification of 80,000 at 100 kV. For the immunogold labeling, membrane-membrane grid was washed with water at room temperature for 10 min and incubated with 5% goat serum in BSA-Tris buffer (20 mM Tris, pH 8.0, 1 mg/ml BSA, 1 mg/ml gelatin, 20 mM NaN3) for 15 min, and treated with primary antibody (25 μg/ml 14D4 monocular antibody, 1% goat serum) for 1 h. Then samples were washed three times with BSA-Tris buffer and incubated with secondary antibodies coupled to 10-nm gold (Amersham Biosciences) for 30 min. They were washed three times with water and negatively stained with 2% uranyl acetate. Images were recorded on FUJI FG film.

HeLa Cell Culture and Transfection Conditions—HeLa cells (ATCC CCL2; American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). For transfection, cells were seeded the day before to ~70% confluency and transfected with the plasmid DNAs described above using LipofectAMINE reagent (Invitrogen). Transfected cells were cultured in 70% confluency and transfected with GFP tagged connexin, fixed cells were incubated for 30 min at 4 °C in 4% paraformaldehyde and 0.1% Triton X-100 with simultaneous quenching with 50 mM glycine and washed with 0.1% Triton X-100 in PBS. For GFP fusion proteins, the coverslips were mounted directly on glass slides, and the fluorescent images was imaged with an LSM Pascal confocal microscope (Zeiss). Co-expression of GFP fused mutant proteins with 14D4-tagged WT connexin, fixed cells were incubated for 30 min at room temperature in blocking buffer (3% BSA, 0.1% Triton X-100 in PBS) before primary antibody ID4 was added for 3 h at room temperature or overnight at 4 °C. Cells were washed with blocking buffer, incubated with Texas Red-conjugated goat anti-mouse antibodies (Molecular Probes) at a dilution of 1:1,000 in blocking buffer for 30 min at room temperature, and imaged with a confocal microscope as described above.

Microinjection and Dye Transfer Analysis—HeLa cells were grown in 35-mm glass bottom dishes and transfected with GFP-tagged connexin. 18 h after transfection, the medium was replaced with PBS, and cells were injected with a 2% solution of the fluorescent dye sulforhodamine (Molecular Probes) at room temperature. Injected cells were chosen by the criteria that GFP-labeled gap junction plaques had formed between neighboring cells. In the case of the Cx43 mutant, cell pairs that were both clearly transfected were chosen. Non-transfected cells were injected as a control. Although Lucifer Yellow is the tracer commonly used to monitor gap junction activity, its fluorescence emission wavelength was too close to the emission wavelength of GFP. To avoid interference, we therefore used sulforhodamine dye instead of Lucifer Yellow to monitor gap junction activity.
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The positions of the three residues Met-34, Cys-64, and Arg-75 in human Cx26 are represented in Fig. 1A, and the Cx26 constructs used in this study are summarized in Fig. 1B. Residue Met-34 is located in the first transmembrane helix (M1) near the extracellular side, and residue Arg-75 is found at the boundary of the first transmembrane loop (E1) and the second transmembrane helix (M2). In addition to the human hearing loss or palmoplantar keratoderma mutants, M34T and R75W (18, 29), we also made M34A, R75A, and R75D substitutions to examine the roles of these two residues more closely. Residue Cys-64 is the third cysteine in E1, and the mutation C64S results in a loss of the electric coupling activity in Cx32 (33). To allow for affinity purification of the expressed proteins, a hexahistidine tag was fused to the C termini of the wild-type and the mutant connexins (named WT-His, M34T-His, etc.). We also produced GFP- and 1D4-tagged fusion proteins (called WT-GFP and WT-1D4), which were used to detect the proteins in transiently transfected mammalian cells. A thrombin-recognition sequence was inserted in all tagged proteins so that the tags could be removed. The total numbers of amino acid residues for the expressed connexins were 238 for WT-His, 471 for WT-GFP, and 248 for WT-1D4.

**Electron Microscopy of Gap Junction Plaques Prepared from Sf9 Cells**—Negative staining technique of electron microscopy was used to check for the formation of gap junction plaques in Sf9 cells expressing WT and mutant Cx26. We isolated membranes from transfected cells and enriched the gap junctions by alkaline extraction (35). Membranes prepared in this way were negatively stained and imaged in the electron microscope. Preparations from Sf9 cells expressing WT connexin frequently showed the typical gap junction plaques consisting of partially ordered hexagonal connexon arrays (Fig. 2A). Plaques of identical appearance were seen at the similar frequency in membrane preparations obtained from Sf9 cells expressing mutant connexins, where residue Met-34 was substituted either by a threonine (Fig. 2B) or an alanine residue (Fig. 2C). The R75A and R75D substitutions had also no significant effect on the appearance and frequency of gap junction plaques (Fig. 2, E and F). Although the R75W substitution also produced gap junction plaques (Fig. 2D), in this case the frequency at which gap junction plaques could be observed was much lower. We found more than 10 plaques per grid square in membrane preparations from Sf9 cells expressing WT, R75A, or R75D connexin, but we only found less than one plaque per grid square in membrane preparations from cells expressing R75W. Although it is difficult to quantify the occurrence of gap junction plaques accurately by electron microscopy, the difference between R75W and all the other connexins was significantly high to reliably conclude that the R75W substitution had an effect on the frequency of gap junction formation. The C64S connexin was the only mutant that did not form gap junctions in our mutations. The expression of the mutant into membranes was confirmed by the immunogold labeling of the alkali membranes (Fig. 2G). In the case of the control Sf9 membranes, no gold particles were observed as shown in Fig. 2H.

When the insect cells, which were maintained in suspension culture, were harvested, there were no signs of cell-cell adhesion. Therefore, the gap junctions seen in the electron micrographs had to be assembled in the ER or other intracellular membranes. Gap junction formation in the ER is a known phenomenon that has already been described with connexins overexpressed in mammalian cells (39). Electron microscopic analysis of freeze-fractured and thin-sectioned specimens confirmed that the gap junction plaques found in ER membranes were similar to those found on the cell surface (39). Therefore, our results were thought to reflect the ability of all mutant connexins with the exception of C64S to adopt the correct tertiary structure and to form gap junction plaques in Sf9 cell membranes.

**Electron Microscopy of Purified Detergent-solubilized Connexons**—A negative staining technique for electron microscopy was used to examine the oligomeric states of mutant connexins expressed in Sf9 cells. To this end, alkaline-extracted membranes were solubilized with 2% dodecyl maltoside (DoDM), and the His-tagged connexins were purified by nickel-affinity chromatography. This one-step purification was necessary to remove impurities that made it difficult to image solubilized connexins in the electron microscope and yielded highly purified protein as shown in Fig. 3. Electron micrographs of negatively stained WT-His, M34T-His, M34A-His, and R75A-His Cx26 revealed mostly homogeneous connexon particles (Fig. 3, A–C and E, respectively), whereas the R75W-His and R75D-His connexins tended to aggregate (Fig. 3, D and F). The C64S-His Cx26 mutant also showed protein aggregates rather than homogeneous particles (Fig. 3G), consistent with the lack of gap junction plaques seen in the membrane preparations as shown in the previous section.

**Gel Filtration Analysis of the Oligomeric State of Mutant Connexins**—To confirm our EM results on the oligomeric states of Cx26, all the purified protein samples were subjected to analytical gel filtration chromatography using a Superose 6 column that was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (230 kDa), bovine serum albumin (68 kDa), and RNase A (13.7 kDa) as standards. For each connexin sample, fractions were collected and analyzed by SDS-PAGE.
FIG. 2. Images of negatively stained membranes isolated from Sf9 cells expressing WT and mutant Cx26. Membranes prepared from Sf9 cells expressing WT (A), M34T (B), M34A (C), R75W (D), R75A (E), and R75D (F) connexin revealed typical gap junction plaques with partially ordered arrays of hexameric connexons. The frequency with which gap junction plaques were seen in the R75W sample was significantly lower than with the other samples. G and H, immunogold labeling of prepared membranes. G, membranes prepared from Sf9 cells expressing the C64S-1D4 mutant connexin were specifically labeled with 10-nm immunogold particles, but no gap junction plaques were observed. H, no gold particles and no gap junction plaques were observed in control membrane preparations isolated from Sf9 samples expressing no Cx26. Scale bars correspond to 50 nm.

FIG. 3. Negative stain preparations of connexins in 2% DoDM. All proteins contained a C-terminal His tag and were purified via nickel-affinity chromatography. Protein purified from Sf9 cells expressing WT (A), M34T (B), M34A (C), and R75A (E) connexin showed typical connexon particles that were stable in 2% DoDM. By contrast, protein purified from Sf9 cells expressing R75W (D), R75D (F), and C64S (G) connexin did not show connexon structures but tended to aggregate. Scale bars correspond to 50 nm.
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Fig. 4. Gel filtration analysis of connexins purified in 2% DoDM. A–G, fractions eluted from a Superose 6 column were analyzed by SDS-PAGE, and the protein bands corresponding to Cx26 are shown here. The numbers at the top indicate the elution fraction. The elution volumes of hexameric connexins and the molecular mass of ~550 kDa and of monomeric connexins of ~90 kDa are shown by a black arrow and a white arrow, respectively. WT (A), M34T (B), M34A (C), and R75A (E) can form the stable hexameric assembly in 2% DoDM solution, whereas only monomeric forms of R75W (D), R75D (F), and C64S (G) are observed in the detergent solution. H–J, SDS-PAGE of the mutants co-expressed with the WT. The mutant proteins were His-tagged, and WT Cx26 was untagged. The nickel affinity-purified connexins were analyzed by gel filtration as above. The upper bands indicate mutant proteins with a tag sequence (black arrowheads), and the lower bands are the WT proteins (white arrowheads). The M34T-His/WT (H) and the R75W-His/WT (I) exhibit the same elution pattern, where the mutant and WT are co-eluted as hexameric connexons at 550 kDa, indicating that the R75W could form stable connexon with the WT. On the other hand, the C64S-His/WT (J) shows a separate elution each other. The protein bands of C64S-His have the peak around 90 kDa (elution volume of 16–17 ml), indicating a monomeric form, and the co-purified WT was in the oligomeric form, which appeared to be smaller than the hexameric assembly. The fractions with the strongest signal for each gel filtration run are indicated by black stars.

Fig. 5. Localization of GFP fusion connexins expressed in HeLa cells. A–G, confocal micrographs of GFP-tagged connexins expressed transiently in HeLa cells. Cells were imaged 18 h after transfection. WT-GFP (A), M34T-GFP (B), M34A-GFP (C), R75W-GFP (D), R75A-GFP (E), R75D-GFP (F) are localized at adjoining plasma membrane areas (arrows). In cells expressing C64S-GFP (G), there was no GFP signal at the plasma membranes, but the signal was spread throughout the cell. R75W-GFP (D) also showed the spread signal inside the cells, but R75A-GFP (E) and R75D-GFP (F) have the puncta spots. H–J, co-expression of 1D4-tagged WT and GFP-tagged mutant connexin in HeLa cells. Cells were fixed 18 h after transfection. Green (GFP) shows the localization of mutant Cx26; red (Texas Red-labeled anti-1D4 antibodies) indicates the localization of WT Cx26, and yellow marks areas where both proteins co-localized. Cells expressing WT-1D4 with either M34T-GFP (H) or R75W-GFP (I) show yellow signal at the plasma membranes, indicating co-localization of the mutant Cx26 with WT (arrows). J, cells expressing WT-1D4 with C64S-GFP revealed only WT-1D4 on the cell surface (red, marked by arrowhead), whereas co-localization of mutant and WT Cx26 (yellow) was restricted to intracellular membranes. Scale bar represents 20 μm.

This substitution is not unexpected, because residue Cys-64 is located in the extracellular domain of Cx26 and is thought to be involved in stabilizing the docking surface of connexons by the formation of a disulfide bond (31). Residue Cys-64 might therefore be crucial in the early phase of gap junction formation, e.g. in the correct folding of the protein, as discussed below.

To test the possibility whether the His tag had an effect on the oligomeric state of wild-type and mutant Cx26, the tags were removed by treatment with thrombin. Because the proteins with or without tag behaved identically on SDS-PAGE and revealed the same connexon structures when observed in the electron microscope (data not shown), we conclude that the
His tag at the C terminus had no significant effect on the properties of the Cx26 mutants used in our studies.

The R75W Connexon in Detergent Can Be Stabilized by Co-expression with WT Connexin—By using analytical gel filtration, we then tested whether the oligomerization of the three mutants, M34T-His, R75W-His, and C64S-His, could be rescued by co-expression of WT connexin. Each His-tagged mutant was individually co-infected with untagged WT Cx26, and the resulting connexin structures were purified by nickel-affinity chromatography. SDS-PAGE analysis of the fractions eluted from the gel filtration column revealed that M34T-His and WT connexin co-eluted in the same fractions, corresponding to a connexin hexamer (Fig. 4H). This result showed that the purified particles were heteromeric connexons containing both WT and M34T connexin. Surprisingly, R75W-His connexin could also form stable hexameric complexes in the presence of WT connexin if an expression ratio of one to one was used (Fig. 4I). This result suggests that the failure of R75W to form DoDM-resistant connexons (Fig. 4D) is not due to aberrant membrane insertion but due to a lack of oligomerization stability of the R75W mutant. This hypothetical conclusion was corroborated by expression of R75W mutant Cx26 in mammalian cells (see below). The C64S-His mutant failed to form stable connexons even in the presence of WT connexin, although an oligomeric structure of co-purified WT connexin could be detected (Fig. 4J). This result indicates that the C64S mutant cannot efficiently oligomerize with WT connexin. A possible explanation for this finding is that C64S mutant Cx26 fails to adopt the correct fold needed for connexon assembly in the membrane.

Expression of GFP-fused Mutant Connexins in HeLa Cells—To study the assembly and channel activity of mutant connexins in mammalian cells, we transiently expressed GFP-fused Cx26 (Fig. 1B) in HeLa cells. Previous studies have shown that fusion of GFP to the C terminus of WT Cx26 has no effect on gap junction assembly or channel function (36). Fluorescence microscopy images of HeLa cells expressing GFP-tagged WT and mutant Cx26 are shown in Fig. 5. All the GFP fusion proteins with the exception of C64S-GFP were trafficked to the adjoining plasma membranes and appeared to assemble into gap junction plaques, consistent with our EM observations using Sf9 cells (Fig. 2). Although R75W and R75D connexons disintegrated upon DoDM solubilization (Fig. 4, D and F), the fluorescence images clearly showed that the proteins were efficiently targeted to adjoining plasma membranes (Fig. 5, D and F), indicating that both mutants retained the ability to correctly insert into membranes and to traffic normally. Only the C64S-GFP mutant was not targeted to the plasma membrane and the green fluorescence signal spread throughout the cell (Fig. 5G). A uniform spreading of GFP signal throughout the cell was also seen with the R75W-GFP fusion protein (Fig. 5D), although it is unclear whether the cytoplasmic GFP signals derived from oligomeric connexins or from misfolded proteins. We also co-expressed the GFP-tagged mutants with WT Cx26 in HeLa cells, as well as in Sf9 cells. For localization of
WT Cx26, a recognition sequence for the monoclonal antibody 1D4 was fused to its C terminus (Fig. 1B), which allowed its detection with a Texas Red-conjugated second antibody against 1D4. The GFP-tagged M34T and R75W mutants co-localized to the plasma membrane (Fig. 5I). These findings are consistent with our results from co-infections of Sf9 cells (Fig. 4, H–J) and suggest that M34T and R75W, but not C64S, connexins can form stable oligomer with WT connexin homotypically and homotypically.

**Dye Transfer Analysis of GFP-fused Connexins in HeLa Cells**—To understand the channel activity of mutant connexins, sulforhodamine, a gap junction permeable fluorescent dye, was injected into HeLa cells expressing GFP-tagged connexins. Although this assay does not provide quantitative measurements of Cx26 channel activities, it is suitable to compare the channel activity of mutant connexins with that of the wild-type Cx26. The dye was injected only into cells showing GFP signal at adjoining membranes. In the case of C64S-GFP, which is not targeted to the plasma membrane, cell pairs were chosen that were both showing GFP signals. Typical images recorded from dye transfer experiments are shown in Fig. 6. Cells expressing WT-GFP or M34T-GFP connexins transported dye across the gap junctions within a few seconds after injection (Fig. 6A and B). By contrast, cells expressing M34A-GFP, R75W-GFP, R75A-GFP, or R75D-GFP Cx26, which were targeted normally to the plasma membrane, exhibited significantly lower channel permeability, and no stain transfer could be seen 60 s after dye injection (Fig. 6, C–F). HeLa cells expressing C64S-GFP connexin also showed no channel activity (Fig. 6G), which is not surprising considering that EM images of these membranes did not show any gap junction plaques.

The numbers of cells transferring dye within 1 min after injection were counted for each GFP mutant (Fig. 7A). More than 80% of the cells expressing WT-GFP or M34T-GFP connexin showed dye transfer, indicating a significant channel activity. Although we did not measure single channel conductance for M34T-GFP connexin, the apparent dye transfer and the normal appearance of gap junctions in Sf9 cells (Figs. 2B and 3B) suggested that M34T connexin causes no profound defect in gap junction channel activity. Nevertheless, a homozygous M34T mutation causes disease. The M34A-GFP, R75W-GFP, R75A-GFP, and R75D-GFP mutants showed reduced channel activities, i.e. only 20–30% of the connexin-expressing cells showed dye transfer, and control unexpressed cells and C64S-GFP mutant cells displayed about 10%. The different activities of the M34T-GFP and the M34A-GFP mutants suggest that the residue at this position might directly affect the channel permeability. In contrast, the loss of channel permeability on Arg-75 mutations might be attributed to the structural instability of the connexons as shown above.

When dye transfer assays were carried out with cells co-transfected with WT-GFP and M34T connexin, the M34T mutant did not interfere with the channel activity of the assembled connexons. However, in cells expressing WT-GFP and R75W connexin, the R75W mutant caused a marked reduction in dye transfer (Fig. 7B). These results correspond well to the clinical phenotypes described for these two mutants, where M34T is a recessive mutation and R75W has a dominant-negative effect (23–27, 29). The results also suggest that although WT connexin can rescue the hexamer formation of R75W (Fig. 5J), the assembled channels are still defective, possibly due to an aberrant channel structure caused by the incorporation of R75W connexins. The C64S mutant did not significantly reduce the channel activity when it was co-expressed with WT-GFP connexin, and about 80% of the cell pairs displayed dye transfer (Fig. 7B). The finding that C64S had no negative effect on WT gap junction channel activity corroborates the finding that C64S connexin is unable to oligomerize or to fold properly. Because C64S-GFP could not be co-localized with WT-1D4 at plasma membrane (Fig. 5J), the channel would be composed of only WT-GFP even when this was co-expressed with C64S. Although we cannot strictly rule out possible artifacts caused by the GFP tags, recent studies (36, 42–44) using GFP-tagged connexins have shown that the GFP tag does not interfere with connexin assembly into fully functional gap junctions.

**Amounts of Protein Expressed in Sf9 Cells**—To understand the difference between M34T mutant and WT connexin, we compared the respective amounts of protein expressed in Sf9 cells by determining the amount of His-tagged protein purified from the same amount of Sf9 membranes (2 mg). M34T-His as well as other mutants reproducibly yielded 2–3-fold higher protein amounts than the wild type (Table I). This difference in protein expression may reflect the fact that Cx26 is a tumor suppressor gene, which down-regulates cell growth (45, 46). Therefore, high expression levels of WT connexin might be toxic for Sf9 cells. The higher expression of M34T-His mutant compared with WT-His connexin might therefore indicate a lower channel permeability for the mutant connexin, although the two proteins appeared to have comparable activities in dye-transfer experiments.

**DISCUSSION**

Our goal was to understand the effect of mutations in three key residues, Met-34, Arg-75, and Cys-64, of human Cx26 on the function of the resulting gap junctions. After exploiting the high level of protein expression in insect cells to visualize the gap junction structures formed by mutant connexins using electron microscopy, we used a mammalian expression system to study the targeting of the mutant connexins to the plasma membrane and to analyze their channel activity. A summary of the results we obtained is given in Table II.

**Table I**

| Purified protein amounts from 2 mg membrane proteins | µg |
|----------------------------------------------------|--|
| WT-His                                             | 30.5 ± 14.6 |
| M34T-His                                           | 59.5 ± 15.3 |
| M34A-His                                           | 82.0 ± 12.1 |

When dye transfer assays were carried out with cells co-transfected with WT-GFP and M34T connexin, the M34T mutant did not interfere with the channel activity of the assembled connexons. However, in cells expressing WT-GFP and R75W connexin, the R75W mutant caused a marked reduction in dye transfer (Fig. 7B). These results correspond well to the clinical phenotypes described for these two mutants, where M34T is a recessive mutation and R75W has a dominant-negative effect (23–27, 29). The results also suggest that although WT connexin can rescue the hexamer formation of R75W (Fig. 5J), the assembled channels are still defective, possibly due to an aberrant channel structure caused by the incorporation of R75W connexins. The C64S mutant did not significantly reduce the channel activity when it was co-expressed with WT-GFP connexin, and about 80% of the cell pairs displayed dye transfer (Fig. 7B). The finding that C64S had no negative effect on WT gap junction channel activity corroborates the finding that C64S connexin is unable to oligomerize or to fold properly. Because C64S-GFP could not be co-localized with WT-1D4 at plasma membrane (Fig. 5J), the channel would be composed of only WT-GFP even when this was co-expressed with C64S. Although we cannot strictly rule out possible artifacts caused by the GFP tags, recent studies (36, 42–44) using GFP-tagged connexins have shown that the GFP tag does not interfere with connexin assembly into fully functional gap junctions.

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**M34T Forms Stable Connexons and Functional Channels**—Previous biochemical studies (22) on rat Cx26 reported that the M34T mutation prevents the efficient assembly of connexins into hexameric connexons. Our studies contradict this finding, and the hexameric stability of M34T was clearly observed in EM and biochemical analysis (Figs. 3B and 4B). We therefore conclude that the M34T mutation in human Cx26 does not interfere with efficient formation of stable connexons. The positive channel activity and recessive functional phenotype of M34T are also inconsistent with the previous studies (21, 22).

Our dye-transfer assays do not provide an absolute measure for single channel permeability and do not rule out the reduced conductance of M34T. It is noteworthy that both M34T and M34A form normal connexons and gap junction plaques, whereas M34A displays significantly reduced channel perme-
The mutation M34T in human Cx32, which is associated with X-linked Charcot-Marie-Tooth disease, also changed the channel properties into mostly substate but the permeability remained with the fully open channel conductance similar to that of the WT (47), providing further evidence that the M34T mutation has no serious effect on the overall structure of the channel. These results prove that residue Met-34 in Cx26 is critical for connexon channel function and might line the pore pathway (Fig. 8A).

The M34-regulating Model for the Connexon Channel—It should be pointed out that the reduction of channel permeability of M34A resulted from a substitution of the original residue with a smaller residue. In the three-dimensional structure of the gap junction, the pore is formed by at least two tilted helices, but the narrowest part of the pore, which is located close to the extracellular side, is formed by only one termed the C helix (15). Oh et al. (47) have demonstrated a decrease in the open channel probability in M34T mutant Cx32 and implicated the contribution of M1 to the pore pathway. Although the reduced permeability may result from subtle conformational defects by the Met-34 mutation, at least the M34T and M34A proteins presented stable connexon structures similar to that of the wild-type even in the 2% DoDM solution (Fig. 3, B and C). Therefore, combining these studies with our results, we propose an M34-regulating model for the fully open state of the gap junction channel, which is schematically drawn in Fig. 8B. In this model, residue Met-34 makes important interactions with other transmembrane helices to stabilize the highly tilted M1 structure and to ensure a favorable open channel state. Reducing the size of the side chain at this position, as in the case of the M34A and M34T mutations, would lead to a partially closed channel, providing a possible explanation for why the substitution of Met-34 with a less bulky residue results in a reduction of channel permeability.

The same effect was also seen in other studies, where the substitution M34C in Cx32E143 and the substitution L35C in Cx46 both exhibited a small but significant decrease in channel conductance (48, 49). It is notable that amino acid residues at position 34 and its flanking region have hydrophobic property. The side chain size as well as its hydrophobicity at their positions could regulate various channel functions in the gap junction.

### Table II

Summary of mutant connexins examined in this study

| Gap Junctions | Hexameric stability | Oligomerization with WT | Targeting | Dye transfer activity | Co-localization with WT |
|---------------|---------------------|------------------------|-----------|----------------------|------------------------|
| WT            | Yes                 | Yes                    | ND                    | Yes                  | 85.7 ± 1.6             | ND                     |
| M34T          | Yes                 | Yes                    | Yes                   | Yes                  | 89.3 ± 2.5             | Yes                    |
| M34A          | Yes                 | Yes                    | ND                    | Yes                  | 26.2 ± 4.8             | ND                     |
| R75W          | Yes                 | No                     | Yes                   | Yes                  | 16.9 ± 2.8             | Yes                    |
| R75A          | Yes                 | Yes                    | ND                    | Yes                  | 15.1 ± 3.1             | ND                     |
| R75D          | Yes                 | No                     | Yes                   | Yes                  | 33.4 ± 2.8             | ND                     |
| C64S          | No                  | No                     | Yes                   | Yes                  | 11.1 ± 2.4             | No                     |

* ND, not determined.
* Low frequency.
* ND, hexameric structure is not rescued.

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Fig. 8. A, model for the location of residues Met-34 and Arg-75 in the connexon structure. A view of connexon perpendicular to the membrane plane is shown. Residue Met-34 is proposed to be located in the innermost helix, where it is positioned close to the next subunit. Residue Arg-75 is proposed to lie on the interface between adjacent subunits, where it contributes to the stabilization of the connexon hexamer. B, Met-34-regulating model. Residue Met-34 in helix M1 of Cx26 is proposed not only to line the pore but also to interact with adjacent subunits ensuring a fully open channel structure. Residues with smaller side chains such as Thr and Ala at this position are insufficient to support the open structure.
tion family. For example, the various residues, such as Met, Leu, and Val (e.g. human Cx26 (37), rat Cx43 (3), and zebrafish Cx43.4 (51), respectively), at this position were identified in native species and might achieve variety in the channel function.

The Arg-75 Residue Is Involved in Inter-subunit Interactions—Newly translated connexons are thought to assemble into connexons on their way from the ER to the plasma membrane (52, 53), and a multiple pathway in the trafficking and assembly routes of connexins has also been reported (41, 53, 54). Our results suggest that the Arg-75 is not important for proper membrane insertion but rather for the stabilization of the connexon structure. We confirmed that co-expression of WT connexin with the R75W mutant rescued the formation of stable connexons (Fig. 4f). This evidence strongly suggests that the Arg-75 is responsible for intersubunit interactions in the connexon (Fig. 8a). The proportion of rescued R75W connexons increased with the amount of co-expressed WT connexin (data not shown), further corroborating the model that the side chain of Arg-75 makes a crucial contribution to the stability of the hexameric connexin assembly. Co-expressed WT connexin in Sf9 cells could not rescue R75D connexin in formation of stable connexons (data not shown). Two mutants at position 75 suggest that positive charge at this position is important for the stabilization of connexons, and negative charge destabilizes the connexon formation. Interestingly, three transmembrane helices, M1, M3, and M4, have a negatively charged glutamic acid residue near the extracellular surface, and the positively charged Arg-75 may form a salt bridge with one or some of these residues to stabilize the oligomeric form. Although the channel activity of R75A is defective, its stable hexameric form implies other interactions to keep the connexon assembly (Figs. 3E and 6E).

The dominant-negative defect of R75W activity is consistent with previous clinical and biophysical reports (29), which is similar to the profile of W44C (55). Interestingly, the substitution of the adjacent conserved residue Trp-77 to an arginine is known to be an autosomal recessive deafness mutant (56). However, this mutant does not form intact connexons and is therefore not targeted to the plasma membrane, so that it does not interfere with channel coupling of WT Cx26 (21, 22, 29, 55). These findings suggest different contributions of Arg-75 and Trp-77 to the gap junction structure. Residue Trp-77 might be important for intramolecular interactions, where W77R causes more profound defects than R75W, resulting in the recessive phenotype. Taken together, the extracellular side in M2 is very important for connexin oligomerization into connexons and stabilization of the gap junction structure.

Cys-64 Plays an Important Role in Connexon Assembly—Although it is commonly accepted that it is the hydrophobic interactions between membrane-spanning regions that are critical for the stabilization of the protein topology, the C64S substitution, which was positioned on an extracellular loop, caused the most profound defects among all the mutations examined. The contributions of the disulfide bonds in the extracellular domain to the structure and function of the connexon channel are complex. Several reports used DTT to reduce the disulfide bonds and to facilitate solubilization with no effect on the stability of the connexons (35, 40). Connexons purified in this way could also be reconstituted into liposomes, and single channel recording revealed normal channel activity (57). Our results suggest that the C64S substitution prevents correct insertion of mutant protein into the membrane. By contrast, an oligomeric WT was co-purified (Fig. 4J), and other studies (33, 34) reported that the cysteine mutants have the ability to insert into membranes. The defects caused by the C64S substitution therefore might be due to inefficient assembly of connexons, similar to the effect of the R75W mutation. The importance of disulfide bonds in the extracellular domains has also been studied in rhodopsin, and the substitutions of cysteines to alanine in rhodopsin resulted in a reduced stability of the activated form (16). In analogy, the conserved extracellular disulfide bridges may be essential in maintaining the functional channel structure in connexins. In any case, our results demonstrate that the residue Cys-64 is of crucial importance in the process of connexon assembly.

In conclusion, by evaluating the capability of Cx26 mutants to form gap junction plaques using an insect cell expression system and electron microscopy, we were able to proposed explanations for the defects caused by substitutions of residues Met-34, Arg-75, and Cys-64. Residue Met-34 is lining the connexon pore, and the size of the side chain at this position determines the channel permeability. We propose that Met-34 is the key residue in the regulation of the channel opening but gives less influence to the connexon assembly. Residue Arg-75 is involved in subunit interactions when connexins assemble into connexons. The transmembrane helix M2 interacts with adjacent subunits, and the interaction might be stabilized via salt bridges including Arg-75. C64S Cx26 cannot form gap junctions or hexameric connexon, but it is able to form a complex with wild-type protein, suggesting that Cys-64 forms an extracellular disulfide bond, which is important to maintain a stable connexin structure that is suitable for connexon formation. Our studies emphasize the fundamental roles of the region from M1 to M2 in Cx26 for the assembly of connexin and for the permeability of the gap junction channel, whereas high resolution structure analysis is required to gain insight into the channel functions.

Note Added in Proof—Another pore lining model of helix3 has been empirically suggested (58).

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