Assembly of the cochlear gap junction macromolecular complex requires connexin 26

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Hereditary deafness affects approximately 1 in 2,000 children. Mutations in the gene encoding the cochlear gap junction protein connexin 26 (CX26) cause prelingual, nonsyndromic deafness and are responsible for as many as 50% of hereditary deafness cases in certain populations. Connexin-associated deafness is thought to be the result of defective development of auditory sensory epithelium due to connexion dysfunction. Surprisingly, CX26 deficiency is not compensated for by the closely related connexin CX30, which is abundantly expressed in the same cochlear cells. Here, using two mouse models of CX26-associated deafness, we demonstrate that disruption of the CX26-dependent gap junction plaque (GJP) is the earliest observable change during embryonic development of mice with connexin-associated deafness. Loss of CX26 resulted in a drastic reduction in the GJP area and protein level and was associated with excessive endocytosis with increased expression of caveolin 1 and caveolin 2. Furthermore, expression of deafness-associated CX26 and CX30 in cell culture resulted in the macromolecular degradation of large gap junction complexes accompanied by an increase in caveolar structures.

Introduction

Hearing loss is the most common congenital sensory deficit (1, 2). Approximately 1 child in 1,000 is affected at birth or during early childhood by severe hearing loss, which is defined as prelingual deafness (3, 4), with about half of the cases attributable to genetic causes (5). Among the more than 100 known forms of nonsyndromic deafness with identified genetic loci, by far the most common and best characterized is the one associated with GJB2 (OMIM 121011), the gene encoding the connexin 26 (CX26) protein (6). This gap junction protein, which assembles to form channels between cells in the cochlear supporting cells, allows the rapid removal of K+ away from the base of hair cells, resulting in the recycling of this ion back to the cochlear supporting cells, allowing the rapid removal of K+ away from the base of hair cells, resulting in the recycling of this ion back to the endolymph to maintain cochlear homeostasis (7). CX26 and CX30 are the two most abundantly expressed gap junction proteins in the cochlea (8) and form heteromeric and heterotypic channels (14). This dynamic process regulates gap junction assembly and disassembly in living cells.

In this study, we demonstrate that a mutation in CX26 induces the macromolecular degradation of large gap junction complexes accompanied by an increase in caveolar structures and that the assembly of this macromolecular complex requires CX26.

Results

In this study, we performed a detailed compositional analysis of cochlear GJPs using models of two major types of CX26-related hearing loss. The first model consists of a mouse that expresses human CX26 with the R75W dominant-negative mutation (CX26(R75W); refs. 15–17 and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI67621DS1). The other is a newly developed conditional Cx26-deficient mouse (Cx26f/f; P0-Cre) with localized gene deletion in the inner ear under the control of the protein 0 (P0) promoter (ref. 18 and Supplemental Figure 1C). To confirm the expression pattern of P0 in the inner ear lineage, P0-Cre mice were crossed with R26R(RFP) reporter mice, which contain GFP knocked into the Rosa26 locus, allowing for the activation of GFP using Cre recombinase, and GFP signals were observed at the otocyst (Supplemental Figure 2). Cx26f/f; P0-Cre mice had severe sensorineural hearing loss (Supplemental Figure 1D), although no abnormalities were observed in other organs (data not shown). Furthermore, these mice displayed an impaired ability to propagate Ca2+ oscillations from cell to cell at P5 (Supplemental Figure 3), which is probably related to an impaired function of gap junctions (19, 20) and

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which occurs before the onset of hearing. We note that although the propagation range was affected, the frequency of the Ca2+ oscillations did not change significantly (Supplemental Figure 3 and Supplemental Videos 4–7).

In a detailed analysis with a three-dimensional graphic construction of the GJP structure in the inner sulcus cells (ISCs; Figure 1A), WT adult mouse cochleae showed large, planar GJPs (L-GJPs; Figure 1I) at the cell border that formed orderly pentagonal or hexagonal outlines around normal ISCs (Figure 1, B, D, F, I, and L). In contrast, cochleae from \textit{CX26R75W+} (Figure 1, C, E, G, J, M, and Supplemental Video 1) and \textit{Cx26 f/f P0-Cre} (Figure 1, H, K, and N) mice showed drastically fragmented, small vesicle–like GJPs (S-GJPs; Figure 1). Drastic disruption of cochlear GJPs in two models of CX26–associated deafness. (A) Schematic illustration of cochlear ISCs. (B and C) CX26 distribution (in red) in ISCs (boxed region in A) in P12 cochlear cryosections from \textit{CX26R75W+} mice show fragmented GJPs (C, small arrows) in contrast to GJPs in control mice (B). (D and E) Double staining of CX26 and CX30 in whole-mount cochlear tissue at 3 weeks showing that these GJPs are composed of both connexins in control (D) and \textit{CX26R75W+} (E) mice. (F–H) GJP formation in 8-week-old adult cochlea from a \textit{CX26R75W+} mouse (G), a non-Tg littermate control (F), and a \textit{Cx26f/f P0-Cre} mouse (H). The partial EGFP signals in G indicate that it is a transgenic animal. (I–K) Image of each boxed region in F–H, respectively. Inset in K shows the GJPs in a \textit{Cx26f/f} littermate control mouse. (L–N) Three-dimensional images reconstructed from the images in I–K, respectively. (O) Lengths of the largest GJPs (brackets in I–K) along a single cell border (mean ± SE, \(n = 25\) for all four groups). *** \(P < 4.7 \times 10^{-14}\) and 1.2 \(\times 10^{-16}\) for \textit{CX26R75W+} and \textit{Cx26f/f P0-Cre} cochleae, respectively, relative to controls (Student’s \(t\) test). (P–R) At E17.5, \textit{CX26R75W+} ISCs showed a number of S-GJPs (Q, arrows), including some L-GJPs (Q, arrowheads), as observed in the non-Tg control ISCs (P). \textit{Cx26f/f P0-Cre} ISCs (R) showed totally disrupted GJPs, although the control ISCs (Cx26f/f, inset in R) showed L-GJPs. Nuclei were counterstained with DAPI (blue). Scale bars: 10 μm.
Changes in gap junction proteins in 8-week-old CX26-mutant mice. (A and B) Immunoblot analysis showed decreased protein expression not only for CX26, but also for CX30 in both CX26R75W+ (A) and CX26R75W P0-Cre (B) mice. CX43 expression in the different cochlear cells was analyzed as a control. (C and D) CX26 and CX30 protein levels were normalized to the corresponding β-actin levels and were expressed relative to the amount present in each littermate control. Values represent the mean ± SEM (error bars; \( P \times 10^{-5} \)) to the cells in all groups. This result indicated the significant reduction in the protein levels of not only CX26 but also CX30 in both CX26R75W+ and CX26R75W P0-Cre mice. CX43 expression in the different cochlear cells was analyzed as a control. (C and D) CX26 and CX30 protein levels were normalized to the corresponding β-actin levels and were expressed relative to the amount present in each littermate control. Values represent the mean ± SEM (error bars; \( P \times 10^{-5} \)).
The hydrophilic region of CAV1α in CX26\[^{R75W}\] cochleae, and found significantly increased expression of CAV1β and CAV2 in both CX26\[^{R75W}\] and Cx26\[^{f/f}\]P0-Cre cochleae (Figure 5, A–D). CAV1 and CAV2 showed excessive accumulation (Figure 5, E–K) in 3-week-old CX26\[^{R75W}\] cochleae. These cells that accumulated CAV1 or CAV2 were occasionally observed at the inter-GJP space or on the surface of the GJPs (Figure 5, G, H, J, and K) in 3-week-old CX26\[^{R75W}\] cochleae, although no accumulated signals were observed in the control mice (Figure 5, E and I). We quantified the number of ISCs that had accumulated CAV1 and CAV2 in both CX26 mutants. There was no difference in their distribution pattern or in the number of positive cells (\(P = 0.16\)) with respect to CAV1 (7.3 ± 1.3%) and CAV2 (5.6 ± 0.9%) accumulation (Figure 5L). In the overexpression of CAV1β with WT CX26, CX26R75W, and CX30 in HEK293 cells, we observed that numerous GJPs at the cell borders and the connexin vesicles were accompanied by accumulated CAV1β, although WT CX26 with CAV1β did not show such a distribution pattern (Supplemental Figure 8). We also observed numerous CAV1- and CAV2-positive vesicles at the S-GJPs in the two mutant mice in the adult stage as compared with those seen at the L-GJPs in the control mice (Figure 5, M and N). These changes were associated with an increase in the caveolar structures (Figure 6, B, E, F, and G–K) that are indicative of endocytosis, which leads to membrane retrieval and, perhaps, abnormal GJP formation.

Based on electron microscopic analysis of the ISCs, we observed that both CX26\[^{R75W}\] and Cx26\[^{f/f}\]P0-Cre mice also formed gap junctions, although there were a number of discontinuous gap junctions (Figure 6, B, D, and K) with excessive endocytosis (Figure 6, B, E, F, and G–K) or abnormally condensed intermembrane layers (Figure 6, E and F) around the gap junctions in Cx26\[^{f/f}\]P0-Cre mice. In contrast with their littermate controls (Figure 6, A and C), significantly larger numbers of caveolae and vesicles were detected at cell borders in both CX26\[^{R75W}\] mice and Cx26\[^{f/f}\]P0-Cre mice (Figure 6L). The distributions and the number of caveolae were nearly identical between both types of mutant mice and were consistent with the immunolabeling for CAV1 and CAV2 around GJPs (Fig-
Figure 4
Disruption of cochlear GJPs is reproduced by human cDNA clones for CX30 and CX26, with or without mutations in HeLa cells, and leads to functional differences in dye transfer depending on the resultant GJP sizes. (A–E) Clear differences in GJP formation were observed in HeLa cells that expressed the indicated connexin(s), which made homomeric or heteromeric channels. Cells were colabeled with anti-CX26 (green) and anti-CX30 (red) antibodies and were counterstained with DAPI (blue). L-GJPs were observed only when normal CX26 was expressed alone (A) or was coexpressed with CX30 (C). The other combinations (B, D, and E) formed S-GJPs. (F–Q) In HeLa cells that expressed CX30 and CX26, cells with smaller GJPs demonstrated decreased NB transfer. (F, G, L, and M) Shown are HeLa cells that expressed WT CX30 alone (CX30) or coexpressed WT CX30 and WT CX26 (CX30/CX26), or the indicated CX26 mutants (CX30/CX26R75Q or CX30/CX26R75W). CX26 and CX30 were colocalized, and the GJP size differed across cell lines. (H and N) Quantitative analysis of the GJP length (mean ± SEM). ***P = 3.3 × 10^{-17} (H) or P = 7.7 × 10^{-12} (N). (I, J, O, and P) Digital fluorescence images of HeLa cells expressing the indicated connexin(s) after NB scrape-loading. (K and Q) Quantitative analysis of intercellular NB transfer after scrape-loading. Columns represent the mean distance (± SEM) of NB transfer from the scrape line. ***P = 9.6 × 10^{-9} (K) or P = 3.2 × 10^{-11} (Q). Scale bars: 10 μm and 5 μm (insets).
ure 5, M and N) and the upregulation of the caveolin proteins (Figure 5, A–D). This may be associated with the membrane retrieval (Figure 6K) caused by excessive endocytosis and the formation of abnormal GJPs. In the labeling with cholera toxin subunit B (CTxB), it was shown that GJPs with CX26 and CX30 did not colocalize with lipid rafts in control mice (Supplemental Figure 9, A, C, and E). In contrast, the lipid raft signals in Cx26f/f P0-Cre mice localized between S-GJPs with no regularity and with more diffuse labeling and less clarity as compared with those in control mice (Supplemental Figure 9, B, D, and F).

Figure 5
Changes in the endocytosis proteins CAV1 and CAV2 in CX26-mutant mouse cochlea. The CAV1 isoform preference shifted from α to β in CX26R75W+ cochlea (A). The CAV1 β and CAV2 increased in both mutant mice (A and C) at 8 weeks of age, as shown by Western blotting. Protein levels of CAV1β (B) and CAV2 (D) are expressed relative to the amount present in each littermate control (mean ± SEM, n = 5). P = 0.009 and 0.03 for CAV1β and CAV2, respectively, in CX26R75W+ cochlea. P = 0.007 and 0.01 for CAV1β and CAV2, respectively, in Cx26f/f P0-Cre cochlea. (E–K) Accumulation of CAV2 and CAV1 in 3-week-old non-Tg littermate controls (E and I) and CX26R75W+ mice (F–H, J, and K) with S-GJPs (brackets in F) was occasionally observed at the inter-GJP space (G, arrow) and on the surface of the GJPs (H, arrowheads), differing from that seen in littermate controls (I and E). The boxed regions in F and J are magnified in G, H, and K, respectively. (L) Numbers of cells with accumulated CAV1 or CAV2 were counted in five animals from each group (mean ± SEM, n = 10; P = 0.0003, 6.1 × 10⁻⁵, 0.001 and 0.002, from left to right). (M–N) In adult mice, vesicles positive for CAV1 (arrowheads) were frequently detected on GJPs in Cx26R75W+ P0-Cre mice (K), but not in their littermate controls (J). Boxed regions are magnified in the bottom right corner. Scale bars: 10 μm and 5 μm (insets). *P < 0.05; **P < 0.01; ***P < 0.001.
with the R75W mutation. We did not, however, observe an effect on GJP formation resulting from disruption of the cytoskeleton (data not shown). As for ubiquitination and autophagy of the GJP, including CX26, we examined the association of ubiquitination and autophagy with GJP disruption in CX26 mutants by immunoblot analysis and transmission electron microscopy. We could not find convincing evidence to show that these processes were more prevalent in CX26-mutant cochleae than in control cochleae (data not shown). As for membrane retrieval at cell junctions, our ultrastructural pathology demonstrated that a significantly larger number of caveolae and vesicles were associated with the cell border (including small gap junctions) in Cx26−/−P0-Cre mice as compared with those in the control mice (Figure 6). This may indicate that excessive endocytosis occurs in the presence of this CX26 mutation, leading to membrane retrieval that may accelerate GJP degradation.

Discussion

In this study, we demonstrated a novel molecular pathology in the cochlea, that of degradation of the gap junction macromolecular complex in CX26-associated deafness, as well as a novel mechanism of GJP formation into one of two types (L-GJPs or S-GJPs) that is dependent on CX26 expression. These findings were consistent not only in cochlear cells, but also in a human cell line with artificial connexin expression. Our data suggest that both the dominant-negative R75W mutation and the absence of CX26 affect the accumulation and assembly of gap junction units in the cell-cell junctions between cochlear supporting cells.

Regarding the initial phenotype of the hearing organ in the connexin mutants (Figure 1 and Supplemental Figure 4), we have previously described an early histological change in CX26-mutant mice, which showed an absence of the small space needed to form the future tunnel of Corti around P5 (15). As for hearing function, the earliest change detected by the auditory brainstem response occurs only after P11 (15), because hearing input to the mouse cochlea normally begins between P11 and P12 (25). With respect to the function of the cochlear gap junction, Ca2+ responses fail to propagate in organotypic cultures with defective expression of CX26 or CX30 from P3 through P6 (26). Thus, to the best of our knowledge, the phenotype at the embryonic stage shown in the present study (Figure 1 and Supplemental Figure 4) has not been previously reported.

The mosaicism generated by our newly developed Cx26−/−P0-Cre mice revealed that the formation of L-GJPs versus S-GJPs critically depended on CX26 expression, and CX26 expression from both adjacent cells was essential to form normal L-GJPs — even if CX30 was highly expressed in the same cells (Figure 1, D and E). The in vivo (Figure 3) and in vitro (Figure 4) findings concerning GJP formation provided conclusive evidence demonstrating that the formation of...
L-GJPs have a definite CX26 dependency and that CX26 is required to form the large macromolecular complex of gap junctions.

Regarding the association between connexins and caveolins (Figure S5 and Supplemental Figure 9), gap junction activity is regulated by protein kinase Cγ activity associated with CAV1-containing lipid rafts (27). Connexin family members (including CX26) are targeted to lipid raft domains, where they interact directly with caveolins such as CAV1 (28). Interestingly, in that experimental system, CX26 was targeted to lipid rafts only when CX26 was coexpressed with CAV1, suggesting that CAV1 recruits CX26 to lipid rafts, although other connexins such as CX32, CX36, CX43, and CX46 showed this lipid raft targeting without any artificial expression of CAV1 (28). These data showing that CX26 is excluded from the lipid raft fraction are in agreement with our present observation that the lipid raft signals in Cx26+/P0-Cre mice localized between S-GJPs with no regularity and with more diffuse labeling and less clarity compared with those in control mice (Supplemental Figure 9). This suggests that the partial targeting to lipid rafts of connexins such as CX30 occurred to prevent the assembly or reassembly of GJPs in CX26-mutant mice.

In many tissues, CX26 is often coexpressed with another connexin in individual cells (29), and it can form heterotypic channels containing homomeric or heteromeric channels with CX30 (10). Hearing loss caused by CX30 deficiency in mice is rescued by the overexpression of CX26 (30). This observation demonstrates that CX26 can compensate for CX30. In contrast, when CX26 is absent in conditional CX26-null mice, CX30 overexpression does not rescue the severe hearing loss (31). Thus, it was concluded that CX26 plays an essential role in the development of the auditory sensory epithelium and that its unique developmental functions required for normal hearing cannot be replaced by CX30 (31). In lens fiber cells, although the expression of CX46 and CX50 overlap extensively, mice that lack one or the other of these connexins (and that serve as a model system for cataract formation) exhibit different phenotypes (32, 33). Macromolecular complex degradation in GJPs that affects other partner connexins and critically reduces functional plaque area may explain not only hearing loss, but also conditions such as cataracts resulting from gap junction dysfunctions with uncompensated connexin mutations.

Until now, hereditary deafness with connexin mutations was believed to be initiated by a single molecular dysfunction or dominant-negative effect on the connexin partners (34), and the primary pathology had never been observed at the embryonic stage. As for the cell degeneration in the connexin mutants, Liang et al. reported that cell degeneration was not a primary cause of CX26 deficiency–associated hearing loss (35). Using a CX26-deficient mouse model similar to the one used in the present study, they demonstrated that cochlear cells, including spiral ganglion neurons, had no significant degeneration throughout postnatal development, although auditory brainstem responses (ABRs) were absent in the whole frequency range (8–40 kHz) after birth.

Here, we demonstrate that connexin-associated deafness, which is the most frequent type of hereditary deafness, may be initiated not by a single molecular dysfunction of connexins, but by a macromolecular complex degradation of GJPs from the embryonic stage. This loss of GJP area may then abolish the proper ionic gradient needed for intercellular communication in the cochlea.

To the best of our knowledge, this is the first report demonstrating that a mutation in CX26 induces macromolecular degradation of large gap junction complexes accompanied by an increase in caveolar structures, which may lead to excessive membrane retrieval and result in a drastic reduction in the GJP area. This may represent a new molecular pathology for hereditary sensorineural deafness and for the general formation of gap junctions, and thus this machinery could be an effective target for drug design and chemical screening to reinforce the assembly of the other cochlear connexins such as CX30.

Methods

Dominant-negative CX26R75W (CX26R75W+) transgenic mice. CX26R75W+ mice were obtained from a breeding colony of a previously reported line (16). CX26R75W+ mice were maintained on a C57BL/6J background and crossed with C57BL/6J animals to generate R75W transgenic offspring. Nontransgenic (non-Tg) littermates on a C57BL/6J background were always used as the control for CX26R75W+ mice.

Generation of novel conditional knockout mouse for CX26. A targeting vector of a floxed Cx26 allele including exons 1 and 2 was constructed using phase DNA clones from a genomic library of J1 ES cells. An approximately 8.4-kb HindIII-BamHI fragment with exon 1 and a 2.85-kb SacI-SacI fragment containing the 3′ half of exon 2 were isolated and used to construct a targeting vector with long and short homologous sequences, respectively. One loxp sequence was introduced at the end of intron 1, and a Neo cassette was introduced between two loxp sequences in exon 2. The diphtheria toxin A (DT-A) chain expression cassette was used as a negative selection marker. The linearized targeting vector was introduced into J1 ES cells by electroporation, and G418-resistant clones were analyzed by Southern blotting to isolate the homologous recombinant as described (18, 36). Identified recombinant ES cells were injected into C57BL/6J blastocysts. A mouse strain harboring the floxed Cx26 allele was established by crossing chimeras with C57BL/6J females to produce F1 heterozygotes (CX26+/−). F2 offspring were generated by crossing F1 heterozygotes, and their genotyping was performed by PCR amplification. No apparent abnormalities were detected in the Cx26+/− mice. Otic vesicle–specific CX26-knockout mice were generated by breeding Cx26+/− mice with mice that expressed the Cre recombinase gene under the control of the promoter of the P0 gene (P0-Cre mice, on a C57BL/6J background), as described (18). To evaluate Cre recombinase expression and distribution, P0-Cre mice were crossed with ROSA26-GFP reporter mice (Gr-ROSA26Sor-13or, R26R), and the four mice from two litters were analyzed by fluorescence and confocal microscopy (Supplemental Figure 2). Cx26−/− on a C57BL/6J background in the littermates was always used as the control for the Cx26−/− P0-Cre mice.

Immunohistochemistry. Mice were anesthetized, killed, and the inner ear tissues were removed. The cochlea were further dissected and fixed in 4% PFA. Immunofluorescence staining with antibodies against CX26 (rabbit IgG from Life Technologies and mouse IgG from LifeSpan Biosciences), CX30 (rabbit IgG; Life Technologies), cavelin 1 (mouse IgG; BD Biosciences), and cavelin 2 (mouse IgG; BD Biosciences) was performed on whole-mount preparations of the finely dissected organ of Corti or cochlear cryosections (5-μm) that included ISCs. We incubated the tissues in the antibody solutions for 1 hour after blocking. The following secondary antibodies were used: Cy3-conjugated anti-rabbit IgG (Sigma-Aldrich) for anti-CX26 or -CX30 rabbit antibodies; Alexa Fluor 488–conjugated anti-mouse IgG (Life Technologies) for anti-CX26 mouse antibodies; Alexa Fluor 594–conjugated anti-mouse IgG (Life Technologies) for anti-CX21 or -CAV2 mouse antibodies; and Alexa Fluor 633–conjugated anti-rabbit IgG (Life Technologies) for CX30 in double or triple staining. Lipid rafts were visualized with Alexa Fluor 594–conjugated cholera toxin subunit B (CtXB, Life Technologies). Fluorescence confocal images were obtained with an LSM510-META confocal microscope (Zeiss). Some of the green fluorescence in CX26R75W+ mice indicates the pseudocolor obtained from
the Alexa Fluor 633–conjugated secondary antibody signal (Life Technologies), because these mice have ubiquitous EGFP expression from their transgene. z-stacks of images were collected at 0.5-μm intervals, and the single image stacks were constructed with the LSM Image Browser (Zeiss); three-dimensional images and videos were constructed using IMARIS software (Bitplane). We analyzed at least five samples from five animals at each age used, and representative images are shown. The compared images were photographed and processed using identical parameters. Three-dimensional images were constructed with z-stacked confocal images by IMARIS (Bitplane). Quantitative analysis of the GJP length (mean ± SEM) was performed with the LSM Image Browser (Zeiss), and data were compared using a Student’s t test (Microsoft Excel).

Western blot analysis. The mouse cochlear proteins were extracted with T-PER Tissue Protein Extraction Reagent (Thermo Scientific) from at least six cochleas that included the organ of Corti, lateral wall, and stria vascularis. The proteins were resolved by SDS-PAGE with 4% to 20% mini-PROTEAN TGX gels (Bio-Rad Laboratories) and then transferred onto a PVDF membrane (Amersham Hybond-P; GE Healthcare). After blocking, the membranes were processed through sequential incubations with anti–caveolin 1 (1:1,000; BD) and anti–caveolin 2 (1:500; Sigma-Aldrich), rabbit anti-CX26 (1:1,000; Life Technologies), mouse anti-CX26 (1:1,000; Life-Span Biosciences), and monoclonal anti–β-actin (1:1,500; Sigma-Aldrich) with HRP-conjugated anti-rabbit or anti-mouse IgG (1:40,000; GE Healthcare) as the secondary antibody. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) was then used for visualization, and the signal was developed on x-ray film (Amersham Hyperfilm ECL; GE Healthcare). Each experiment was repeated at least three times. Densitometric analysis of the band intensities was performed with ImageJ software (NIH). The data were normalized to the corresponding β-actin levels, relative to the amount present in each littermate control, and compared using a Student’s t test (Microsoft Excel).

Transmission electron microscopy. Animals were deeply anesthetized and perfused intracardially with 0.01 M PBS, followed by 2% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer. The cochleas were opened and flushed with the fixative for 2 hours at room temperature. After washing, the specimens were postfixed for 1.5 hours in 2% osmium tetroxide in 0.1 M phosphate buffer and then dehydrated through a graded ethanol series and embedded in Epon. Horizontal sections of the surface of the cochlear membrane labyrinth were cut, stained with uranyl acetate and lead citrate, and examined by electron microscopy (H-7100; Hitachi). The numbers of caveolae and vesicles were counted and compared using a Student’s t test (Microsoft Excel).

Expression constructs and cell lines expressing CX26, CX30, or CX26 mutants. Expression constructs and cell lines were generated as previously described (10, 22). Briefly, human GJB6 was obtained by RT-PCR (Superscript II; Life Technologies) from human corpus callosum RNA (Clontech) and subcloned into the pIRESpuro3 vector, then human GJB2 was subcloned into the pIRESneom3 vector, and the GJB2 mutations were introduced into the ORF of human GJB2 cDNA by PCR site-directed mutagenesis using the QuickChange kit (Stratagene). Communication-incompetent HeLa cells were used to generate the stable cell lines expressing WT CX30. To generate cells expressing both WT CX30 and WT CX26 or CX26 mutants, one cloned cell line that stably expressed CX30 was transfected with GJB2 in pIRESneom3. After selection with both 1 μg/ml of puromycin (Sigma-Aldrich) and 1 mg/ml of G418 (Life Technologies) for about 3 weeks, the colonies were trypsinized, and these bulk-selected cells were expanded for the subsequent studies. Quantitative analysis of GJP length (mean ± SEM) was performed with ImageJ software.

Immunocytochemistry and GJP quantification. HeLa cells were grown on coverslips for 2 days to a confluence of approximately 70% to 90%, fixed in acetone, and incubated with a monoclonal antibody (33-5800, diluted 1:500; Zymed Laboratories) and a rabbit antiserum against the C terminus of CX30 (71-2200, diluted 1:1,000; Zymed Laboratories); these did not cross-react with each other, as previously shown (10). The cells were visualized with tetramethylrhodamine isothiocyanate–conjugated (TRITC-conjugated) donkey anti-rabbit (Abcam) and FITC-conjugated donkey anti-mouse secondary antibodies (Abcam). The cells were photographed under a Leica fluorescence microscope with a Hamamatsu C4742-95 digital camera connected to a G5 Mac computer using OpenLAB 2.2 software for deconvolution. The images were analyzed for GJP length using ImageJ software. All GJPs from 30 to 35 cells of each cell line were measured, and the mean GJP length was compared between cells expressing CX30 alone and those expressing both CX30 and CX26, or between cells expressing both CX30 and CX26R75Q and those expressing both CX30 and CX26R75W, using a Student’s t test (Stata).

Dye transfer with scrape-loading. Dye transfer was investigated using a scrape-loading assay as previously described (10). Briefly, parental HeLa cells, bulk-selected cells that expressed WT CX30 alone, or cells that coexpressed WT CX30 and CX26 or one of the CX26 mutants (R75Q or R75W) were grown to confluence on coverslips. Following the scrape-loading with 2% NB and diffusion, the cells were washed, fixed, and the NB was visualized by TRITC-conjugated avidin (Sigma-Aldrich). Cells were photographed under a Leica fluorescence microscope with a Hamamatsu C4742-95 digital camera connected to a G5 Mac computer, using OpenLAB 2.2 software. Dye transfer was quantified by measuring the distance from the scrape line to the point where the average fluorescence intensity dropped to 1.5 times the background intensity. Eleven to twelve images were acquired from each of three different plates of cells. The images were processed and analyzed with ImageJ software, and the mean distance was calculated using Microsoft Excel software and compared using a Student’s t test (Stata).

Statistics. A one-tailed Student’s t test, with a significance criterion of P < 0.05, was used to compare the GJP length, relative protein levels, number of cells positive for accumulated caveolins, ABH thresholds, propagation ranges of Ca2+ signaling, distance of dye transfer, and number of caveolae or vesicles.

Study approval. All experimental protocols were approved by the IACUC of Juntendo University School of Medicine and were conducted in accordance with the NIH guidelines for the care and use of laboratory animals.

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