Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca\textsuperscript{2+} signaling than homomeric counterparts

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Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca\textsuperscript{2+} signaling than homomeric counterparts. Am J Physiol Cell Physiol 288: C613–C623, 2005; doi:10.1152/ajpcell.00341.2004. —The importance of connexins (Cxs) in cochlear functions has been demonstrated by the finding that mutations in Cx genes cause a large proportion of sensorineural hearing loss cases. However, it is still unclear how Cxs contribute to the cochlear function. Recent data (33) obtained from Cx30 knockout mice showing that a reduction of Cx diversity in assembling gap junctions is sufficient to cause deafness suggest that functional interactions of different subtypes of Cxs may be essential in normal hearing. In this work we show that the two major forms of Cxs (Cx26 and Cx30) in the cochlea have overlapping expression patterns beginning at early embryonic stages. Cx26 and Cx30 were colocalized in most gap junction plaques in the cochlea, and their coassembly was tested by coimmunoprecipitation. To compare functional differences of gap junctions with different molecular configurations, homo- and heteromeric gap junctions composed of Cx26 and/or Cx30 were reconstituted by transfections in human embryonic kidney-293 cells. The ratio imaging technique and fluorescent tracer diffusion assays were used to assess the function of reconstituted gap junctions. Our results revealed that gap junctions with different molecular configurations show differences in biochemical coupling, and that intercellular Ca\textsuperscript{2+} signaling across heteromeric gap junctions consisting of Cx26 and Cx30 was at least twice as fast as their homomERICALLY assembled counterparts. Our data suggest that biochemical permeability and the dynamics of intercellular signaling through gap junction channels, in addition to gap junction-mediated intercellular ionic coupling, may be important factors to consider for understanding functional roles of gap junctions in the cochlea.

GAP JUNCTIONS facilitate intercellular communications by providing conduits linking the cytoplasm of attached cells. Each gap junction is an intercellular channel consisting of a pair of connexons in apposed cell membranes that align with the help of multiple disulfide bonds in the intercellular space. Connexons are made of six protein subunits called connexins (Cxs). Connexons are defined as homomeric when the six Cxs are identical or heteromeric when two or more isoforms of Cxs are coassembled. Twenty different Cx genes have been found in the human genome so far, and nineteen have been identified in that of the mouse (38). The protein products of Cx genes all possess four membrane spanning regions, two extracellular loops, and three cytoplasmic segments, including the NH\textsubscript{2} and COOH termini. Protein sequences of different Cxs differ mainly in the COOH-terminal tail and cytoplasmic loop. Cells that form gap junctions usually express more than one subtype of Cx, and many are known to be able to coassemble into functional gap junctions. These diverse molecular arrangements give rise to distinct permeability required for ionic and biochemical transfer through gap junctions suited for particular types of tissue (8).

The importance of Cxs in normal cochlear functions has been demonstrated by many genetic studies showing that about one-half of inherited childhood nonsyndromic deafness cases are caused by mutations in the Cx26 gene (21, 27, 28). Less frequently encountered are mutations in other Cx genes, e.g., Cx30 (10), Cx31 (22, 40), and Cx32 (2). It is clear that mutations in Cx genes are one of the most common forms of human genetic defects resulting in hearing loss for millions of patients with either autosomal recessive or dominant deafness (7, 17). The role of gap junction and the interactions of different subtypes of Cxs in the cochlea are currently unknown. Still less apparent is why mutations in Cx genes, which are widely expressed in a variety of tissues, can be mostly nonsyndromic. It has been speculated that gap junction networks in the cochlea provide intercellular conduits by which potassium ions are recycled from the base of hair cells to the endolymph (18, 32). However, experimental results directly testing this hypothesis are currently not available.

To understand how gap junctions contribute to the intercellular communication in the inner ear, it is essential to investigate the distribution and molecular assembly of different subtypes of Cxs in the cochlea. Earlier freeze-fracture studies have suggested an abundant presence of gap junctions in the inner ear (15, 16). Immunolabeling results demonstrated widespread expression of Cx26 and Cx30 in the supporting cells of the sensory epithelia, fibrocytes in the spiral ligament and spiral limbus, and also in vestibular systems (18, 19). Our recent findings showed that Cx26 and Cx30 are the two most abundantly expressed Cx subtypes, and that they coassemble to form gap junctions in the adult cochlea (1). However, it is not clear how extensive this coassembly occurs temporally and spatially in the cochlea, and whether wild-type gap junctions with different molecular configurations may exhibit functional differences. In this study, we first investigated coexpressions of Cx26 and Cx30 in the cochlea during development. The coassembly of Cx26 and Cx30 in most gap junctional plaques in the cochlea was shown by coimmunostaining and confirmed

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by coimmunoprecipitation. We further quantitatively compared differences in the kinetics of intercellular Ca\(^{2+}\) signaling across gap junctions reconstituted in vitro with either homo- or heteromeric configurations. Results demonstrated that heteromERICALLY assembled gap junctions consisting of Cx26 and Cx30 had the advantage of allowing Ca\(^{2+}\) signaling to spread intercellularly twice as fast as their homomERICALLY assembled counterparts.

**MATERIALS AND METHODS**

Tissue preparation, immunofluorescent labeling, and coimmunolabeling of Cx26 and Cx30 on cochlear cryosections. Mice (strain CD-1, either sex) from embryonic day 14 to adult were used in this study. In some experiments we also used transgenic mice carrying enhanced green fluorescent protein (eGFP) under the control of an enhancer from the Math1 gene (Math-1-eGFP mice). These mice were obtained from Dr. Jane Johnson of the University of Texas Southwestern Medical Center (14). The experimental protocol for animal use was approved by the Institutional Animal Care and Use Committee of the House Ear Institute. Cochlear tissues were fixed in 4% paraformaldehyde prepared in phosphate-buffered solution (PBS; pH 7.4) overnight at 4°C after they were dissected out carefully using microdissecting tools operated with the aid of a stereomicroscope. Ossified cochleae were decalcified in 120 mM EDTA in PBS for 10 days, and then embedded in a small volume of 10% gelatin dissolved in water for at least 2 h at room temperature. The embedded samples were cut into small blocks (<3 mm cubes) and cryoprotected by submerging in 2.3 M sucrose solution overnight (at 4°C) in an Eppendorf tube fixed on an orbital rotor. The immunolabeling results shown in Figs. 1 and 2 were obtained from cochlear sections cut by a conventional cryosectioning machine (model CM1900; Leica Microsystems, Bannockburn, IL) at a thickness of 8 μm. More detailed analyses of Cx colocalization in gap junction plaques by coimmunolabeling (Fig. 3) were obtained from much thinner cryosections cut with an ultramicrotome (Leica-Ultracut UCT; Leica Microsystems) usually used for preparing samples for electron microscopy. Cryochamber of the ultramicrotome was cooled to −60°C before sectioning was started, and the semithin sections were cut at a thickness of 0.5 μm, with the cutting head of ultramicrotome cooled to −120°C.

Polyclonal antibodies, generated in rabbit and mouse monoclonal antibodies against Cx26 and Cx30 (Zymed Laboratories, S. San Francisco, CA), were used for labeling of cochlear sections. No cross-reactions were observed between the two antibodies. The single band showed on Western blots of cochlear tissues corresponded to the expected molecular weight of Cx26 and Cx30 (Fig. 4, A and B). Furthermore, the two antibodies recognized only the corresponding Cx-eGFP fusion proteins on the Western blots of membrane proteins obtained from transfected HEK-293 cells (Fig. 4, D–F) (eGFP different dilution factor in Fig. 4G). These results confirmed the specificity of the Cx26 and Cx30 antibodies used in our studies. The sections were permeabilized and blocked for nonspecific antibody binding with donkey serum (20%, vol/vol) and bovine serum albumin (2%, vol/vol) dissolved in PBS (pH 7.4) containing 1% Tween 20. They were then labeled with antibodies against Cx26 (1:200 dilution) and/or Cx30 (1:200 dilution) overnight at 4°C. After being washed three times with PBS, the sections were labeled with appropriate

Fig. 1. Immunolabeling results of connexin (Cx)26 and Cx30 obtained from cochlear sections cut at various developmental stages indicated by labels at top left corner of each panel. Cryosections were cut at a thickness of 8 μm. White brackets indicate the approximate boundary of the developing organ of Corti. RM, Reissner’s membrane; SL, spiral limbus; P2–P12, stages of hearing development. Scale bars at bottom left corner of each panel represent ~100 μm.
secondary antibodies conjugated to either Cy2 or Cy3 (1:500 dilution; Jackson Immunolab, West Grove, PA). Cochlear sections were also counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:500 dilution) for 5 min (Molecular Probes, Eugene, OR) before they were mounted in an antifade medium (Molecular Probes) and examined using a confocal microscope (model LSM; Zeiss, Shrewsbury, PA). Negative controls were processed similarly with primary antibodies substituted by an equal volume of PBS. Colocalization of Cx26 and Cx30 in gap junction plaques was quantified in specific subregions of the cochlea from at least four different cryosections. Results are given in Table 1.

Coimmunoprecipitation and Western blot analyses of gap junctions obtained from cochlea and transfected cells. Membrane proteins were extracted using a proteoExtract native membrane protein extraction kit (Catalog No. 44810; Calbiochem, San Diego, CA). Cochlear tissues of 10 mice or two dishes of HEK-293 cells (cultured on 60 mm dishes 48 h posttransfection) were homogenized with a Dounce tissue grinder (Fisher Scientific, Tustin, CA). The tissues were then washed twice in ice-cold wash buffer and incubated with extraction buffer I for 10 min at 4°C. Protease inhibitor cocktail (PIC; 10 μl) was added to prevent the activity of proteases released from the cells. Cells were then incubated with 1 ml of ice-cold extraction buffer II supplemented with 10 μl PIC for 30 min at 4°C under gentle agitation. The cell suspension was centrifuged at 16,000 g for 15 min at 4°C, and supernatant containing membrane proteins was collected. Protein concentrations were measured by using a bichinchoninic acid protein assay kit (Pierce, Rockford, IL). For immunoprecipitation, primary antibody against Cx30 diluted at 1:250 was added to the membrane protein extract and incubated overnight. Antibody and connexon complexes were purified by protein A-linked sepharose beads using a protocol as described earlier (6). The protein complex was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) and detected by Western blot analysis using Cx26 and/or Cx30 antibodies (1:1,000 dilution, 4°C), as described in RESULTS. For quantitatively comparing the expression levels of Cxs after transfection of Cx26 and Cx30 constructs were obtained from Dr. Howard Evans (Department of Medicine, Biochemistry and Diagnostic Radiology, University of Wales College of Medicine) and Dr. Willecke (Institut für Genetik, Bonn, Germany), respectively. The Cx coding sequences were cut out from the multiple cloning sites (MCSs) and ligated into the MCSs of pEGFP vector (BD Biosciences, Palo Alto, CA) to create Cx-eGFP fusion protein (Cx-eGFP) after transfections. We also ligated Cx coding sequences into the pIRE2-DsRed2 vector (BD Biosciences) to create separate Cx and DsRed proteins (Cx-IRE2-DsRed) in the same cell. Transfecting HEK-293 cells with the Cx-eGFP and Cx-IRE2-DsRed constructs together allowed us to estimate the efficiency of cotransfection. We also checked colocalization and coassembly of Cx26 and Cx30 in the same reconstituted gap junction plaques (Figs. 4 and 5). Growth medium [DMEM+10% fetal bovine serum + penicillin (100 IU) + streptomycin (100 μg/ml)] was replaced with OptiMEM (Invitrogen, Carlsbad, CA) before transfection. The transfection protocol for cells grown in 60-mm culture dishes is described below. Reagent amount and volume were adjusted according to the surface area of the dish when other types of dishes were used. After all reagents were warmed to room temperature, DNA (8 μg) and Plus reagent (16 μl) were mixed in 500-μl final volume of OptiMEM. For cotransfection, two different plasmids (4 μg each) were mixed in the same tube. In a separate tube, 7.5 μl of Lipofectamine was diluted in the same volume of OptiMEM (500 μl). After 30 min, the contents of both tubes were mixed together to allow DNA and Lipofectamine complex formation by incubation at room temperature for another 20 min. This complex was applied dropwise to the culture dish in a final volume of 5 ml. The dishes were swirled to distribute the complex evenly and returned to a CO₂ incubator (5%, 37°C). After 3 to 4 h of transfection, 500 μl of fetal bovine serum were added to the dish to reduce the toxicity of Lipofectamine and incubation was continued overnight. The transfection medium was replaced 15 h later with the regular growth medium.

Measurements of intercellular Ca²⁺ signaling through reconstituted gap junctions and fluorescent dye diffusion assay. Cells were normally bathed in the Hanks’ balanced salt solution (HBSS; Sigma). In our experiments cell pairs forming gap junctions were identified directly by the presence of GFP. For measuring intracellular Ca²⁺ concentration ([Ca²⁺]), cells were loaded with fura-2 AM (5 μM; Molecular Probes) by incubating with the Ca²⁺ indicator dye for 15 min at room temperature. One cell in the pair was stimulated mechanically by slightly touching the cell membrane with a microelectrode (tip size ~1 μm) mounted on a micro-engine.
manipulator (model MP-285; Sutter Instrument, Novato, CA). The procedure for maneuvering the micropipette was similar to that used to form the cell-attached recording configuration in patch-clamp recordings. Intercellular Ca\(^{2+}\)/H\(_{11001}\) signaling through purinergic receptors was blocked by the addition of suramin (200 \(\mu\)M) (Figs. 7C and 8) or saturated by adding 20 \(\mu\)M ATP (Fig. 7D) in extracellular solutions. The standard ratio imaging technique (11) was used to monitor \([\text{Ca}^{2+}]_{i}\) changes in the stimulated cell (cell 1 in Fig. 7) and those cells surrounding it. Each region of interest was drawn to include the entire cell to measure the average intracellular changes in \([\text{Ca}^{2+}]_{i}\). Experiments were carried out on a Zeiss Axiovert 135 inverted microscope (Zeiss) equipped with a Polychrome II calcium ratio imaging system (TILL Photonics). \([\text{Ca}^{2+}]_{i}\) changes over a period of time were fitted with an exponential equation: 

\[
[\text{Ca}^{2+}]_{i}(t) = \frac{(A_1 - A_2)}{[1 + e^{(t - \tau)/\sigma}]} + A_2, \ 
\]

in which \(A_1\) and \(A_2\) are resting and steady-state \([\text{Ca}^{2+}]_{i}\), \(\tau\) is time point to reach half plateau and \(\sigma\) time constant describing the rising \([\text{Ca}^{2+}]_{i}\). We used fluorescent dyes, propidium iodide (PI), and AlexaFluor 488 for the fluorescent dye diffusion assay. The two dyes have similar molecular weight but opposite charges. These cell-impermeable dyes were injected into single cells by single-cell electroporation based on the technique described by Haas (12).

RESULTS

Expression patterns of Cx26 and Cx30 in the cochlea overlapped from early stages of cochlear development. Multiple types of Cxs are found by immunolabeling in the mature cochlea, including Cxs 26 (18), 30 (19), 31 (9, 39), and 43 (9, 20). Detected at the mRNA level, Cx26 and Cx30 are the two most abundantly expressed subtypes (1). We expanded the previous work by examining the expression patterns of Cx26 and Cx30 at various developmental stages in the cochlea. Immunolabeling results were obtained from E14 to adult cochlear cryosections with antibodies against Cx26 or Cx30 (Fig. 1). Western blot analysis results indicated that the two antibodies had no cross reactivities in recognizing Cx26 and Cx30 (Fig. 4, A, B, and D). The only band shown on the gel had the expected molecular weight of corresponding Cxs in either
native or fusion protein configurations (Fig. 4), which confirmed the specificity of the antibodies used in our studies (Fig. 4, C and E–G). From E14.5 and until before birth (Fig. 1, developmental stage indicated at top left corner of each panel), cells expressing Cx26 and Cx30 were localized both medially and laterally abutting the developing organ of Corti (Fig. 1 and Fig. 2A, brackets indicate the approximate location of the organ of Corti), roughly corresponding to the nascent spiral limbus (arrowheads in Fig. 1) and lateral wall (arrows in Fig. 1). Between E14.5 and E18.5, Cx expressions were completely absent in the sensory epithelia of the developing organ of Corti, including both sensory and supporting cells (brackets in Fig. 1 and Fig. 2A). This is further confirmed by using cochlear sections obtained from Math1-eGFP mice. In this line of mice hair cells are specifically marked by GFP (Fig. 2), as indicated by brackets. Western blot detection of cochlear proteins immunoprecipitated with Cx30. Both antibodies against Cx26 and Cx30 were used in the Western blot analysis. D: Western blot detection of Cx30 using the membrane fraction of proteins extracted from transfected HEK-293 cells. E and F: Western blots of membrane proteins extracted from transfected HEK-293 cells and immunoprecipitated with antibody against Cx30. Antibodies against either Cx26 (E) or eGFP (F) were used. G: Western blots of the membrane protein extracted from HEK-293 cells after cotransfection with Cx26 and Cx30 (left lane), transfection with Cx30 (middle) or Cx26 (right) alone. The amount of loading in each lane was shown by immunoblot of actin, a housekeeping protein.

Fig. 4. Western blot and coimmunoprecipitation results of Cx26 and Cx30 obtained from cochlear tissues (A–C) and human embryonic kidney (HEK)-293 cells transfected with Cxs (D–H), as indicated by brackets. Western blots of Cx26 (A) and Cx30 (B) in the membrane fraction of cochlear proteins as indicated at bottom of each data panel. C: result of Western blot detection of cochlear proteins immunoprecipitated with Cx30. Both antibodies against Cx26 and Cx30 were used in the Western blot analysis. D: Western blot detection of Cx30 using the membrane fraction of proteins extracted from transfected HEK-293 cells. E and F: Western blots of membrane proteins extracted from transfected HEK-293 cells and immunoprecipitated with antibody against Cx30. Antibodies against either Cx26 (E) or eGFP (F) were used. G: Western blots of the membrane protein extracted from HEK-293 cells after cotransfection with Cx26 and Cx30 (left lane), transfection with Cx30 (middle) or Cx26 (right) alone. The amount of loading in each lane was shown by immunoblot of actin, a housekeeping protein.

Table 1. Colocalization of Cx26 and Cx30 in gap junction plaques in various regions of the cochlea

<table>
<thead>
<tr>
<th>Cochlear Regions</th>
<th>Overlapped Cx26 (%)</th>
<th>Total Cx26 Spots Counted</th>
<th>Overlapped Cx30 (%)</th>
<th>Total Cx30 Spots Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hansen and Claudius cells</td>
<td>97±10</td>
<td>188</td>
<td>82±9</td>
<td>223</td>
</tr>
<tr>
<td>Around IHCs</td>
<td>96±12</td>
<td>76</td>
<td>91±11</td>
<td>80</td>
</tr>
<tr>
<td>Deiter cells</td>
<td>94±7</td>
<td>21</td>
<td>14±6</td>
<td>143</td>
</tr>
<tr>
<td>Spiral limbus</td>
<td>98±8</td>
<td>403</td>
<td>94±5</td>
<td>422</td>
</tr>
<tr>
<td>Lower SL</td>
<td>84±12</td>
<td>145</td>
<td>95±14</td>
<td>129</td>
</tr>
<tr>
<td>Middle SL</td>
<td>98±6</td>
<td>159</td>
<td>85±7</td>
<td>183</td>
</tr>
<tr>
<td>Upper SL</td>
<td>96±9</td>
<td>181</td>
<td>89±8</td>
<td>198</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 cryosections. Cx, connexin; IHC, inner hair cells; SL, spiral limbus. Percentages of gap junction plaques coimmunostained were calculated based on data collected from seven cochlear regions (see Fig. 3). Four cryosections were used to obtain the averaged data.
overlapping. One notable exception was the Deiter cells under
the outer hair cells, where most gap junction plaques (86%)
showed immunoreactivity for Cx30 only.

Each gap junction plaque contains hundreds of gap junction
channels; therefore, colocalization of Cx26 and Cx30 in the
similar gap junction plaque demonstrated by immunolabel-
ing does not prove that the two proteins are assembled together
into the same gap junction. Direct interactions of Cx26 and
Cx30 by coassembling into the same gap junction were exam-
ined by coimmunoprecipitation. The experimental procedures
were repeated eight times, and the same results were obtained.
As pointed out earlier, the specificity of the antibodies we used
against Cx26 and Cx30 was confirmed using Western blot
analysis (Fig. 4, A and B). Membrane proteins isolated from
cochlear tissues (P1 mouse) were immunoprecipitated with the
antibody against Cx30, and the precipitated protein complex
was analyzed in the Western blots using antibodies against
both Cx26 and Cx30 (Fig. 4C). Results consistently yielded
two bands corresponding to Cx26 and Cx30, suggesting that
Cx26 and Cx30 physically interact with each other in the
cochlea. To test whether Cx26 and Cx30 were coassembled in
reconstituted gap junctions after transfections, we immunopre-
cipitated membrane protein extracted from co-transfected
HEK-293 cells with the antibody against Cx30. Physical inter-
actions of Cx26 with Cx30 were supported by subsequent
Western blot analysis results showing that 1) Cx26 was present
in the Cx30 immunoprecipitated protein complex (Fig. 4E); 2)
two bands matching Cx30-eGFP and Cx26-eGFP fusion pro-
teins were present if an antibody against eGFP was used in the
Western blots of the Cx30 immunoprecipitated proteins (Fig.
4F). We compared the gap junction protein levels in the cell
membrane by immunoblotting with an antibody against eGFP
(Fig. 4G), which is common to both Cx26- and Cx30-eGFP
fusion proteins produced with transfections. Loading differ-

Fig. 5. Demonstration of cotransfection and coimmunolab-
eling of Cx26 and Cx30 in HEK-293 cells after transfe-
tions using both Cx26-IRESDsRed-eGFP and Cx30-eGFP DNA
constructs. Cotransfection is shown by overlapping fluores-
cent signals of DsRed (A) and GFP (B) from individual
cells. Colocalization of the two Cxs in the same reconsti-
tuted gap junction plaque was demonstrated by immunola-
beling Cx26 (C) to compare with the eGFP-tagged Cx30 (D).

Fig. 6. Permeability of reconstituted gap junction
channels with different molecular configurations
(indicated by labels at top left) evaluated by fluo-
rescent dye diffusion assay. AlexaFluor 488 (top)
and propidium iodide (PI; bottom) were used. Ar-
rows indicate the presence of reconstituted gap junc-
tions shown by eGFP at the cell membrane border-
ing the two cells. Asterisks mark the injected cells in
the cell pair tested.
ences in each lane were controlled by detecting the amount of a housekeeping protein (actin, Fig. 4H). Regarding transfections done with a single type of Cx [Cx30-eGFP (middle lane) and Cx26-eGFP (right lane) in Fig. 4G], a comparison of the intensities of the bands showed that co-transfection using half of the plasmid for each Cx (left lane, Fig. 4G) did not increase the total number of gap junctions in the cell membrane. Total amount of connexin in cotransfectant was comparable to that when only one type of connexin was used in transfections.

The results presented above showed that expressions of Cx26 and Cx30 were remarkably similar both temporally and spatially during cochlear development, and that the Cxs physico-interacted with each other in cochlear gap junctions and reconstituted gap junctions in HEK-293 cells. Combining these results and our earlier demonstration that Cx26 and Cx30 co-assembled in reconstituted gap junctions in HEK-293 cells. Combining these results and our earlier demonstration that Cx26 and Cx30 were remarkably similar both temporally and spatially during cochlear development, and that the Cxs physico-interacted with each other in cochlear gap junctions and reconstituted gap junctions in HEK-293 cells. Combining these results and our earlier demonstration that Cx26 and Cx30 co-assembled in reconstituted gap junctions after cotransfections. Among them, ∼5% of cell pairs formed gap junctions visible by bright GFP spots on the cell membrane (Fig. 5, C and D, Fig. 6, and Fig. 7A). Only the cell pairs that showed clear gap junction formation were used for functional studies of gap junctions. Cotransfection of the two Cxs in the same cell was confirmed by using Cx26-IREDS-DsRed and Cx30-eGFP plasmids together for transfections (Fig. 5, A and B). Simultaneous presence of red (DsRed, Fig. 5A) and green (eGFP, Fig. 5B) fluorescence in the same cell was observed in almost all cells we counted from random views of three dishes (96%, n = 128). We also checked co-localization of Cx26 and Cx30 in the same gap junction plaques by immunolabeling (Fig. 5, C and D). Results demonstrated that Cx26-labeled gap junction plaques (Fig. 5C, arrow) overlapped with eGFP-tagged Cx30 (Fig. 5D, arrow) in most cell pairs we checked (89 of 91). The coimmunoprecipitation results presented above (Fig. 4, D–F) further confirmed that Cx26 and Cx30 co-assembled in reconstituted gap junctions after cotransfections.

To investigate whether gap junctions with different molecular configurations show any difference for intercellular signaling, we first compared their permeability to two charged fluorescent dyes. The two fluorescent dyes we used have similar molecular weights [668 for propidium iodide (PI) and 643 for AlexaFluor 488] but opposite valences. We found that the Cx26WT (Fig. 6D), Cx30WT (Fig. 6E), and Cx26&30WT GFP 24 h after transfecting cells with Cx-eGFP constructs.
Fig. 6F) gap junctions were all readily permeable to the positive charge PI (n = 18). In contrast, homomeric Cx30WT gap junctions were not permeable to the negatively charged AlexaFluor 488 (Fig. 6B). The other two types of reconstituted gap junctions were readily permeable to AlexaFluor 488 (n > 10 for each test) (Fig. 6, A and C). More functional differences between the three types of wild-type gap junctions were shown by quantitatively comparing the rate of intercellular Ca\(^{2+}\) signaling through Cx26WT, Cx30WT, and Cx26&30WT gap junctions. Slight deformation of the cell membrane with a microelectrode (tip size ~1 μm) always elicited rapid increases in [Ca\(^{2+}\)], in the touched cell (Fig. 7, cell 1 or cell touched). Increases of [Ca\(^{2+}\)], spread to neighboring cells apparently by two mechanisms: 1) by propagating transient [Ca\(^{2+}\)], increases mediated by purinergic receptors; 2) by gap junction-mediated intercellular signaling. The purinergic receptor-mediated intercellular signaling was easily distinguishable from that mediated through gap junctions by their longer time delay of onsets and more transient responses. Purinergic receptor-mediated [Ca\(^{2+}\)] responses in surrounding cells usually returned to baseline completely in ~50 s (Fig. 7B, data curves encircled by curved arrow), compared with >300 s needed for gap junction-mediated Ca\(^{2+}\) responses to return (Fig. 7, B–D). In addition, adding suramin (200 μM, Fig. 7C) to block or adding ATP (20 μM, Fig. 7D) to saturate purinergic receptors eliminated the purinergic receptor-mediated intercellular signaling. The suramin and ATP treatments, however, left the gap junction-mediated intercellular Ca\(^{2+}\) signaling intact (Fig. 7, C and D). Only the cell formed gap junctions with the mechanically stimulated cell, followed with an increase in [Ca\(^{2+}\)], (cell 2 in Fig. 7, C and D). All other surrounding cells that did not form gap junctions with the source cell showed no increases in [Ca\(^{2+}\)], (data curves encircled by curved arrows in Fig. 7, C and D). These results indicated that the Ca\(^{2+}\) signaling spread intercellularly through the reconstituted gap junction channels could be studied separately from that mediated by the purinergic receptors.

Rising phases of [Ca\(^{2+}\)] increases in both source (Fig. 7A, cell 1) and follower (Fig. 7A, cell 2) cells could be well fitted by single exponential increases (red smooth curves in Fig. 7E). The Ca\(^{2+}\) signaling between the two cells was characterized by three parameters: 1) a time delay in the onset of Ca\(^{2+}\) rises in the follower cells (dT); 2) a faster time constant (τ1) and a much slower time constant (τ2) in the curve fits describing the [Ca\(^{2+}\)] increases in the cell touched and cell followed, respectively; 3) ratio of the amplitude of peak [Ca\(^{2+}\)] rises in the cell touched (PI) and cell followed (P2). Figure 8 presents results comparing the three parameters among the Cx26WT, Cx30WT, and Cx26&30WT gap junctions. Three panels in Fig. 8A give examples of exponential curve fits to the three types of reconstituted gap junctions with different molecular configurations, and another panel gives raw data of time constants (τ1 and τ2) gathered from all cell pairs we tested. While no significant differences were found for τ1, τ2S fitted for Cx26WT (solid squares) and Cx30WT (open triangles) gap junctions were significantly slower than those of Cx26&30WT gap junctions (Fig. 7A, open circles). The averaged results given in Fig. 8B showed that τ1 (in ms) of three types of gap junctions were 284.5 ± 14.9 (n = 15), 275.9 ± 11.3 (n = 13), and 305.6 ± 19.8 (n = 12) for Cx26WT, Cx30WT, and
Comparison, the homomeric Cx26 or Cx30 were 3,011, these rate differences (dT and $n$) in the follower cells. The dT were (in ms) $1,268 \pm 205$ ($n = 15$), $1,045 \pm 186$ ($n = 13$), and $574 \pm 102$ ($n = 12$) for Cx26WT, Cx30WT, and Cx26&30WT gap junctions, respectively. In addition, the time constant describing the [Ca$^{2+}$]$_i$ increases in the follower cells ($\tau_2$) formed heteromeric gap junctions with the source cell was $1,197 \pm 67$ ($n = 12$). In comparison, the $\tau_2$ values for gap junctions consisting of homomeric Cx26 or Cx30 were $3,011 \pm 196$ ($n = 15$) and $2,758 \pm 153$ ($n = 13$), respectively. Both dT and $\tau_2$ for the intercellular Ca$^{2+}$ signaling across heteromeric gap junctions were about half of those obtained from the homomeric gap junction channels. As demonstrated by results shown in Fig. 4G, these rate differences (dT and $\tau_2$) were unlikely to be caused by increased number of gap junction channels after cotransfections of Cx26 and Cx30.

**DISCUSSION**

Heteromeric connexons have been reported in many types of tissues, including liver (6), lens (37), mammary glands (23), and smooth muscle cells (13). Diversity in gap junction molecular assembly is essential for the specific needs in particular tissues, as it appears that the function of each subtype of Cx usually is not redundant (36). This is most convincingly demonstrated in the lens of the eye, where it is clear that the appropriate heteromeric assembly of Cx46 and Cx50 is crucial for the clarity and growth of the lens (25). Genetic mutation (29) and immunolabeling data (18, 19) both suggest the presence of multiple types of Cxs in the inner ear. Cx26 and Cx30 apparently are the two most abundantly expressed Cxs in the cochlea (1). Lower levels of either mRNA or protein expressions of Cx29 (1), Cx31 (9, 39), Cx43 (1, 5, 9, 20), Cx45 (5), and Cx50 (9) are either detected or shown by LacZ reporter expressions in the inner ear. The presence of multiple types of Cxs suggests functional redundancy and coassembly of compatible Cxs in the inner ear. In this work we expanded the previous work (1, 9, 19) by using a multidisciplinary approach to study the temporal and spatial variations of coassembly of Cx26 and Cx30 in the cochlea. Furthermore, we investigated the functional differences of gap junctions resulting from changes in molecular configurations.

Cxs are classified into at least three subgroups based on their sequence similarities. Cxs26, 31, and 30 belong to the β group, Cxs43 and 50 belong to the α group, and Cx29 belongs to the putative ε group. Generally Cxs from different subgroups are not able to form functional heterotypic gap junctions (3). Thus gap junction-mediated intercellular communications are restricted among clusters of cells expressing compatible Cxs. Intercellular communication along a selective pathway can thus result in a compartmentalization for intercellular signal exchanges within the cochlea. Cx31 was found in a subpopulation of fibrocytes near spiral prominence where both Cx26 and 30 are not present (a region indicated by asterisks in Fig. 1) (9). Although the exact locations of Cx43 and Cx50 expressions are currently controversial (5, 9, 20), it is clear that they do not generally overlap with Cx26 and Cx30. Cx45 was expressed in the inner ear vascular system (5). Expression pattern of Cx29 in the cochlea has not been checked yet. However, it is generally found in myelin-forming glial cells (31). We have shown new data in this work demonstrating that both Cx26 and 30 were expressed early in development and that their expressions in the cochlea were developmentally regulated. The expression of the two Cxs was limited to regions outside the developing organ of Corti before birth and gradually increased in both supporting cells and fibrocytes in the lateral wall. Our result demonstrates for the first time that a band of cells bordering stria vasularis expressed high levels of both Cx26 and Cx30 before the onset of hearing in mice. This pattern of Cx expression changed to a more homogeneous distribution in the lateral wall after P12 (Fig. 1). This dramatic change in Cx expression patterns may reflect an underlying transformation in the demand for both biochemical and ionic couplings in the maturing cochlea. In the adult stage both Cx26 and Cx30 were found in many parts of the cochlea along the proposed K$^+$ recycling pathway (18, 32). Therefore, alteration of their functions is expected to have severe consequences for ionic or biochemical homeostasis. On the other hand, the maintenance of cochlear homeostasis requires appropriate driving force in terms of chemical or electrical potential gradients in the right amount and direction, and directional rectifications of gap junction to help both ionic and biochemical fluxes to go in the appropriate direction. Presence of multiple types of Cxs may help to fine tune the gap junction channels both temporally and spatially appropriate for the physiological demands of the cochlea.

Our finding that most gap junction plaques along the proposed K$^+$ recycling pathway in the cochlea were formed by co-assembly of Cx26 and Cx30 raises some interesting issues. Co-assembly of Cx26 and Cx30 indicates redundancy in building gap junction intercellular channels, and suggests that deleting one Cx gene does not necessarily eliminate gap junction channels in the cochlea. Instead, homomeric gap junctions might be able to replace heteromeric gap junctions where they are normally found in the cochlea. Indeed, the expression pattern of Cx26 in the cochlea of Cx30 knockout mice is indistinguishable from that in the wild-type mice (33). Homomeric gap junctions constituted by either Cx26 or Cx30 have good permeability to K$^+$ (24, 34); therefore, it is puzzling why such a seemingly subtle change (at least for ionic permeation) resulted in deafness and reduction in endolymphatic potential in mice (33). One hint is that gap junctions consisting of homomeric Cx26 or Cx30 have different permeation profiles for certain larger molecules, despite the fact that the two Cxs share the most similar sequences in the Cx family. It is known that gap junctions consisting of homomeric Cx30 are not permeable to Lucifer yellow (LY; molecular weight = 457 atomic mass unit, charge = −2), whereas homomeric Cx26 gap junctions allow LY to pass readily (24). We expanded this line of work by showing that all three types of gap junctions we studied were permeable to propidium iodide, which is a fluorescent tracer molecule with a molecular weight of 668 and a charge of +2. In contrast, permeability of heteromeric gap junctions measured by diffusion of AlexaFluor 488 was similar only to that of homomeric Cx26 gap junctions.
that AlexaFluor 488 and propidium iodide have similar molecular weight (643 vs. 668 atomic mass unit) but different charges (−3 vs. +2), the results suggested that the pore of heteromeric gap junction channels in the cochlea might filter out negatively charged molecules more selectively than positively charged molecules. It was recently suggested that affinity binding sites may exist in the pores of gap junctions to facilitate passage of specific molecules (35). This model of gap junction permeation suggests that gap junctions may not act like inert and passive channels as traditionally believed.

Besides possible differences in biochemical permeability, our data demonstrated a clear distinction between heteromeric and homomeric gap junctions with regard to their efficiency in spreading intercellular Ca2+ signals. Results showed that [Ca2+]i increases in the source cell passed only to neighboring cells formed wild-type gap junctions with the source cell to reach an eventual equilibrium (P2/P1 in Fig. 8B). The Ca2+ signaling did not spread to those cells that formed no gap junctions (Fig. 7, C and D) or nonfunctional mutant gap junctions (e.g., Cx26Δ75W; data not shown) with the source cell. These results assured us that we had measured gap junction-mediated intercellular Ca2+ signaling. Shorter time delay (dT) of [Ca2+]i rises in the follower cells through heteromeric gap junction channels also suggested that changes in gap junction molecular assembly, but not the total number of gap junction channels, were responsible for kinetic alterations.

Transfers of a variety of signaling molecules through gap junction channels have been investigated using cellular imaging methods similar to those used in this study. Previous work shows that mechanical stimuli quickly increase [Ca2+]i and Ca2+ rises propagate to neighboring cells by either simple diffusion of Ca2+ or secondarily as a result of IP3 diffusion through gap junctions (4, 30). It is unclear which signaling molecule (or both of them) crossed gap junctions in the current study. However, based on our observations that 1) we can fit [Ca2+]i, rises in the follower cells (cell 2 in Fig. 5) with a single exponential rising curve and 2) the average amplitude of mechanically elicited Ca2+ responses obtained in Ca2+-free HBSS were less than one-half of those obtained in normal HBSS (data not shown), we believe that direct transfer of Ca2+ across the reconstituted gap junction channels constituted a significant source for the increase in [Ca2+]i in follower cells. Regardless of which molecules are the major ones to cross the gap junctions, faster intercellular Ca2+ signaling shown by heteromerically assembled gap junctions suggested that transient behaviors of intercellular exchanges through gap junctions of different molecular assembly may be an important factor to consider for the function of gap junctions in the cochlea. In addition, our finding suggests that, in addition to recycling K+, gap junctions in the cochlea may have other vital roles in transiently regulating the needs for intercellular communications.

Both Cx26 and Cx30 are found in many different types of tissues; therefore, it is unclear why Cx mutations mostly cause nonsyndromic deafness. If heteromeric assembly of Cx30 and Cx26 is unique to the cochlea and the gap junction properties of this particular combination are vitally suited to the demands of homeostasis in the cochlea, this could provide one simple explanation for the nonsyndromic feature of human Cx26 mutations found in most patients. Our demonstration that heteromeric gap junctions consisting of Cx26 and Cx30 mediated intercellular Ca2+ signaling faster than their homomeric counterparts is consistent with this theory. Alternatively, different tissues may have different tolerance thresholds for quantitative changes in gap junction-mediated intercellular ionic or biochemical couplings. The cochlea, with its unique ionic environment in fluid compartments tightly coupled to mechanotransduction, may happen to be the most sensitive to gap junction mutations. Heteromeric assembly of Cx26 and Cx30 also has some functional implications for Cx mutation studies. It suggests that Cx26 mutations, recessive or dominant, must have transdominant effects on gap junction channels composed of Cx26 and Cx30. This was recently confirmed by others (26).

In this work we studied gap junction-mediated Ca2+ signaling through gap junctions of different molecular configurations as an example of functional specificity of native gap junctions in the cochlea. Considering the diversity in the clinical syndrome of many Cx26 mutations, much remains to be discovered to answer the special biophysical properties and functional roles of gap junctions in the cochlea.

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