Pannexin1 Channels Contain a Glycosylation Site That Targets the Hexamer to the Plasma Membrane

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Pannexins are newly discovered channel proteins expressed in many different tissues and abundantly in the vertebrate central nervous system. Based on membrane topology, folding and secondary structure prediction, pannexins are proposed to form gap junction-like structures. We show here that Pannexin1 forms a hexameric channel and reaches the cell surface but, unlike connexins, is N-glycosylated. Using site-directed mutagenesis we analyzed three putative N-linked glycosylation sites and examined the effects of each mutation on channel expression. We show for the first time that Pannexin1 is glycosylated at Asn-254 and that this residue is important for plasma membrane targeting. The glycosylation of Pannexin1 at its extracellular surface makes it unlikely that two oligomers could dock to form an intercellular channel. Ultrastructural analysis by electron microscopy confirmed that Pannexin1 functional areas do not appear as canonical gap junctions. Rather, Pannexin1 channels are distributed throughout the plasma membrane. We propose that N-glycosylation of Pannexin1 could be a significant mechanism for regulating the trafficking of these membrane proteins to the cell surface in different tissues.

Intercellular communication takes place in two different ways: release of molecules such as neurotransmitters, hormones, or ATP; and formation of intercellular channels that connect directly the cytoplasm of adjacent cells. Multicellular organisms have evolved to use different types of intercellular channels, called gap junctions. In vertebrates, a gap junction channel is formed by the apposition of two hexameric assemblies of connexins (Cx), one from each adjacent cell. A new class of proteins analogous to connexins in their folding, but completely unrelated in sequence has been identified in invertebrates. These proteins were named innexins (innexin analogs, abbreviated as Inx). Despite the conserved structure, properties, and functions of gap junctions in vertebrates and invertebrates, connexin and innexin structures evolved independently.

Mammalian analogs of innexins were recently identified in the human genome sequence and the term pannexins (from the Greek word pan, “throughout ” and the Latin nexus, “connection,” abbreviated as Panx) was coined to name this new class of proteins (1). Pannexins have some sequence similarity to invertebrate innexin proteins, but share no homology with vertebrate connexins. It has been speculated that there is an evolutionary relationship between pannexins and innexins. Both groups of proteins are predicted to have four transmembrane regions, two extracellular loops and intracellular amino (NH₂) and carboxyl (COOH) termini. The human pannexin family consists of three members: Pannexin1 (Panx1, 426 amino acids, 47.6 kDa), Pannexin2 (Panx2, 664 amino acids, 73.3 kDa), and Pannexin3 (Panx3, 392 amino acids, 44.7 kDa). Pannexins are expressed in many different systems and abundantly in the vertebrate central nervous system. Based on membrane topology, folding and secondary structure prediction, pannexins have been proposed to form gap junction-like structures (1). Earlier studies by Bruzzone et al. (2) in paired Xenopus oocytes found that the expression of Panx1, alone and in combination with Panx2, induces intercellular channels. However, functional and localization studies in erythrocytes, cultured neurons, and glia have shown only plasma membrane staining with no evidence of canonical gap junctions (3, 4). One current hypothesis is that Panxs do not duplicate the function of Cxs in connecting directly the cytoplasm of adjacent cells, but rather connect the cytoplasm to the extracellular space. The hemichannels formed by Panx1 in the plasma membrane have been shown to be mechanosensitive ATP conduits (5) that respond to several signal transduction pathways. They are involved in the release of small molecules, and may promote the propagation of calcium waves (6–10).

In this study, we investigated the stoichiometry and membrane targeting of Panx1 oligomers. Panx1 is widely expressed in several tissues, and the expression pattern varies in different locations. For example, Panx1 revealed postsynaptic localization in rodent hippocampal and cortical principal neurons (11). Furthermore, Panx1-specific labeling was evident in the perinuclear region of retinal ganglion cells and horizontal cells, whereas relatively little plasma membrane staining was observed in neonatal retinal ganglion cells, gradually increasing with age (12). To uncover the mechanism regulating the local-
ization of pannexins, in particular subcellular compartments, might provide insights into the functional role of these novel channel proteins in different tissues. The data presented here show for the first time that Panx1 forms a multimeric channel and contains a glycosylation site at its extracellular surface that is important for the trafficking of the protein to the cell surface. The presence of a glycosylation site argues that the single hexamer is the primary channel state in contrast to connexin-based structures that are formed by two docked hexamers where each hexamer can act as an independent unit (13).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—We used the following primary antibodies: chicken anti-Panx1 (4515, characterized in Ref. 3); mouse monoclonal anti-Myc (Sigma); mouse anti-α-tubulin (Sigma); and rabbit polyclonal anti-calnexin (Stressgen, Victoria, Canada).

**Plasmids and Mutagenesis**—The cDNAs encoding rat Panx1 wild-type, myc-tagged were kindly provided by Dr. Roberto Bruzzone. Site-directed mutagenesis was done with the QuikChange kit (Stratagene, La Jolla, CA) using *Pfu* Turbo DNA polymerase. Complementary pairs of synthetic oligonucleotides containing the desired mutation were used to amplify the full-length plasmid with the rPanx1 cDNA insert. The PCR product was treated with DpnI endonuclease to digest the methylated wild-type DNA template, and transformed into MAX Efficiency DH5α Competent Cells (Invitrogen). Successful incorporation of the mutations was confirmed by DNA sequence analyses, using forward and reverse primers to check the entire rPanx1 sequence, and confirm the absence of random mutations.

**Cell Culture and Transfections**—HEK-293T cells were maintained at 37 °C, and 10% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen). Plasmids (0.5 μg for transfection of 35-mm dish) were transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen). Transfected cells were processed 1–2 days post-transfection.

**Triton X-100 Solubilization of Tissue Culture Cells**—Adherent HEK-293T cells were washed 3 times in ice-cold incubation buffer (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.35 mM KH₂PO₄, 0.8 mM MgSO₄, 2.7 mM CaCl₂, 20 mM HEPES, 200 mM phenylmethylsulfonyl fluoride, pH 7.4, and filtered on a 0.22-μm filter), scraped, and centrifuged at 11,500 × g for 10 min at 4 °C. The cellular pellet was resuspended in incubation buffer (1 ml/confluent 60-mm dish), sonicated (3 pulses, 30 s each, on ice), and supplemented with Triton X-100 to a final concentration of 1%. The sample was incubated on ice for 30 min, vortexing 3–4 times during the incubation. The extract was centrifuged at 301,000 × g for 1 h at 4 °C in a SW55 rotor in a Beckman ultracentrifuge. The supernatant was recovered and stored on ice for the cross-linking procedure.

**Chemical Cross-linking**—We used the homobifunctional, amine-reactive reagent DSP (dithio-bis(succinimidylpropionate)) to covalently link Panx1 monomers within the oligomer. The DSP solution was prepared just before use (12.5 mg/ml in Me₂SO), and added to a final concentration of 50 or 300 μg/ml, vortex mixed, and incubated 30 min at room temperature. The reaction was stopped by adding 20 μl/ml of 1 M glycine, pH 9.2, vortex mixed, and incubated on ice for 30 min. A solution of 10 μl/ml of 1 M glycine, pH 7.2, was added to restore the pH. The samples were analyzed by 4–20% SDS-PAGE, 4–12% Tris glycine, or 3–8% Tris acetate gradient gels and Western blot with anti-Myc antibody.

**Gel Electrophoresis and Immunoblotting**—Western blot were performed as described by Towbin et al. (14). Proteins were extracted from cells in SDS buffer containing 4% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma), separated on a 4–20% Tris glycine gel (Invitrogen), and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked overnight in 5% nonfat dry milk and 0.5% Tween 20 in phosphate-buffered saline, and incubated for 1 h with primary antibody: chicken anti-Panx1 (1:1000), mouse anti-α-tubulin (1:7000), or mouse monoclonal anti-Myc (1:5000). Blots were developed with an ECL kit (Amersham Biosciences). The intensity of signals was quantified using the ImageJ software (rsb.info.nih.gov/ij).

**Analysis of Glycosylation and Phosphorylation**—Cell lysates from HEK-293T cells expressing Panx1 wild-type or mutant proteins were incubated with 1,500 units of endoglycosidase H (Endo H) or 1,500 units of N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) for 3 h at 37 °C at pH 5.5 and 7.5, respectively; or with 10 units of calf intestinal alkaline phosphatase (CIAP) for 3 h at 37 °C.

**Immunocytochemistry and Confocal Microscopy**—Cells were grown on poly-D-lysine-coated glass coverslips, fixed with 4% paraformaldehyde/phosphate-buffered saline for 20 min, washed, and labeled for immunofluorescence. The primary antibodies (mouse anti-Myc 1:200, and rabbit polyclonal anti-calnexin 1:2000) were mixed and diluted with blocking buffer. Secondary antibodies (fluorescein isothiocyanate-conjugated anti-mouse and Cy5-conjugated anti-rabbit) were diluted 1:100 in the same buffer. Image processing employed an Olympus Fluoview1000 laser-scanning confocal microscope (Olympus, Center Valley, PA). Image analysis of z-scan was done using the Imaris software (Bitplane AG).

**Cell Surface Biotinylation**—HEK-293T cells were washed three times with Hanks’ balanced salt solution containing 0.05% sodium azide. Cells were incubated at 4 °C with 0.25 mg/ml of Sulfo-NHS-SS-Biotin (Pierce) for 30 min. To confirm that the biotinylation reagent did not label intracellular proteins, the experiment was conducted in 2 blots in parallel, one was hybridized with the chicken anti-Panx1 antibody and the other with the mouse anti-α-tubulin antibody.

**In Vivo Labeling with ReAsH-EDT₂ and FlAsH-EDT₂**—MDCK cells stably expressing a tetracysteine domain (FLNC-CPGCCCMC)-tagged rPanx1 (rPanx1-4C) (15), or rCx43-GFP-4C were labeled for 1 h at 37 °C with 0.5 μM ReAsH-EDT₂ or 0.9 μM FlAsH-EDT₂, 12.5 μM EDT in Hanks’ balanced salt solution. Free and nonspecifically bound ReAsH or FlAsH was removed by washing with EDT (600 μM, 20 min at 37 °C in Hanks’ balanced salt solution). Cells were then incubated for several hours (chase time) in the presence of complete medium. The newly synthesized recombinant proteins were stained at the end of the chase time with the second label, which was...
DUCTANCE was determined with the dual voltage clamp tech-

ony of the junctional conductance was performed by pairing the oocytes. Junctional conductance was removed with forceps and the oocytes paired. Junctional conductance was not observed even at 300 μM DSP, which is an amino-reactive reagent. At a high concentration of DSP (300 μg/ml) two bands were clearly observed (Fig. 1B). Based on size, they are likely to correspond to the rPanx1-Myc dimeric (98 kDa) and hexameric forms (~290 kDa). At a lower concentration of DSP (50 μg/ml) a ladder of partially cross-linked products was observed including the monomer (49 kDa), dimer (98 kDa), trimer (147 kDa), and hexamer (~290 kDa) (data not shown). Breaking the cross-links by boiling in the presence of 5% β-mercaptoethanol resulted in the disappearance of the intermediate forms (Fig. 1B), reducing all products to the monomer (49 kDa). The contrary, boiling in the absence of β-mercaptoethanol did not reverse the cross-links and the intermediate products were observed (data not shown). Bands higher than the corresponding hexamer were not observed even at 300 μg/ml of DSP indicating that the cross-linker DSP allowed the formation of an oligomeric complex and not an aggregate. To further confirm this result, the cross-linked sample was run on a 3–8% Tris acetate gel that allows better resolving of very large proteins, using HiMark Pre-stained HMV Protein Standard as reference. As shown in Fig. 1C, the higher band mapped just above the 268-kDa marker band indicating it corresponds to the Panx1 hexameric form (~290 kDa). Under these conditions, besides the hexamer and dimer bands, we also observed the monomer as a single band.

RESULTS

Panx1 Forms a Multimeric Channel—The oligomeric state of a protein can be analyzed using chemical cross-linkers, as it had been shown for connexins (20). To test whether Panx1 forms a multimeric channel, we treated Triton X-100-solubilized extracts of HEK-293T cells expressing rPanx1-Myc (49 kDa) with DSP, which is an amino-reactive reagent. At a high concentration of DSP (300 μg/ml) two bands were clearly observed (Fig. 1B). Based on size, they are likely to correspond to the rPanx1-Myc dimeric (98 kDa) and hexameric forms (~290 kDa). At a lower concentration of DSP (50 μg/ml) a ladder of partially cross-linked products was observed including the monomer (49 kDa), dimer (98 kDa), trimer (147 kDa), and hexamer (~290 kDa) (data not shown). Breaking the cross-links by boiling in the presence of 5% β-mercaptoethanol resulted in the disappearance of the intermediate forms (Fig. 1B), reducing all products to the monomer (49 kDa). The contrary, boiling in the absence of β-mercaptoethanol did not reverse the cross-links and the intermediate products were observed (data not shown). Bands higher than the corresponding hexamer were not observed even at 300 μg/ml of DSP indicating that the cross-linker DSP allowed the formation of an oligomeric complex and not an aggregate. To further confirm this result, the cross-linked sample was run on a 3–8% Tris acetate gel that allows better resolving of very large proteins, using HiMark Pre-stained HMV Protein Standard as reference. As shown in Fig. 1C, the higher band mapped just above the 268-kDa marker band indicating it corresponds to the Panx1 hexameric form (~290 kDa). Under these conditions, besides the hexamer and dimer bands, we also observed the monomer as a single band.

FlAsH-EDT₂ for the cells initially labeled with ReAsH-EDT₂ or vice versa. After washing, cells were either fixed with 4% paraformaldehyde (for light microscopy imaging) or 2% glutar-
aldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for EM processing.

Photoconversion of Diaminobenzidine and Electron Microscopy—After 20 min fixation, cells were rinsed in cacodylate buffer and blocked for 30 min on ice with 5 mM KCN, 20 mM aminotrizole, 50 mM glycine to reduce nonspecific background. Photoconversion and EM preparation were performed as previously described (16).

Scrape Dye Loading/Transfer Assay—MDCK parental and stably expressing rPanx1-wt, rPanx1-4C, or rCx43-4C were grown on poly-D-lysine-coated glass coverslips. The procedure for these assays follows el-Fouly and colleagues (17); however, after 4% paraformaldehyde fixation, samples were processed for immunofluorescence.

Electrophysiology—The oocyte cell-cell channel assay was performed as described earlier (18). The follicle layer was removed from oocytes by collagenase treatment. The oocytes were injected with in vitro transcribed Panx1 or Cx46 cRNA and incubated for 2–3 days. The vitelline membrane was removed with forceps and the oocytes paired. Junctional conductance was determined with the dual voltage clamp technique (19) at various time points after pairing.
lysates (Fig. 2C, lane 1) suggesting that phosphorylation is not responsible for the multiple bands of Panx1.

To test whether the multiple band pattern could represent different glycosylated species, a glycosidase treatment of rPanx1-WT or rPanx1-Myc protein was performed using Endo H and another enzyme, PNGase F, which does not distinguish between the differentially processed N-linked glycosylated sugars. As shown in Fig. 2C, after Endo H treatment only the intermediate band shifted suggesting this band corresponds to high mannose-type glycoproteins residing in the ER (Fig. 2C, lane 3). The upper and lower bands were Endo H insensitive. PNGase F treatment down shifted both upper and intermediate bands suggesting that the higher band represented the fully processed protein (mature-type glycoprotein, trans-Golgi glycosylated) expressed on the cell surface (Fig. 2C, lane 4). The lower band was not affected by PNGase F treatment, suggesting it corresponded to the non-glycosylated core protein. Similar results were obtained in another stably expressing cell line (MDCK rPanx1-4C) where the higher band was sensitive to PNGase treatment and the lower one was not (data not shown).

In summary, rPanx1 is N-glycosylated and forms three species: GLY0 (the non-glycosylated core protein), GLY1 (the high mannose-type glycoprotein), and GLY2 (the fully processed glycoprotein).

Preventing Glycosylation of Pannexin1 by Site-directed Mutagenesis Results in Intracellular Localization—We analyzed the amino acid sequence of rPanx1 for putative N-glycosylation sites (CBS prediction server, NetNGlyc, Ref. 23). We found four predicted N-glycosylation sites at asparagine 204, 254, 337, and 423. Asn-204, Asn-337, and Asn-423 are located in the predicted cytoplasmic domains, whereas Asn-254 is in the predicted transmembrane topology of rPanx1 identified from sequence analysis. Asn-254 is fully glycosylation-deficient. The relative densities of the different forms of protein (GLY0, non-glycosylated core protein, GLY1, the high mannose-type glycoprotein, and GLY2, the fully processed glycoprotein) were quantified and represent the average value of three to seven independent Western blots ± S.E.
the second extracellular loop of rPanx1. To determine which of the predicted sites is important as a glycosylation site, we used site-directed mutagenesis to inhibit N-glycosylation. Two intracellular and one extracellular asparagine (Asn-204, Asn-337, and Asn-254, respectively) were mutated to glutamine (Fig. 3A). The Asn-423 had a lower prediction score than Asn-337 and because this residue is at the distal end of the C terminus it seemed unlikely that it would be a glycosylation site. The Asn to Gln mutants were transiently expressed in HEK-293T cells and examined by Western blot analysis and immunofluorescence.

As shown in Fig. 3B, the N204Q mutant was similar to the WT protein as it showed all different species of protein (GLY0, GLY1, and GLY2); conversely, the N254Q mutant was fully glycosylation-deficient as it lacked both the intermediate (GLY1) and upper (GLY2) bands (Fig. 3C) and was resistant to PNGase F treatment (data not shown). Furthermore, the N337Q mutant was only partially glycosylated as it showed the intermediate band (GLY1), but lacked the upper (GLY2) band (Fig. 3C). The immunocytochemical localization of wild-type, Myc-tagged and mutant proteins was compared with that of the ER residing protein calnexin. Both Myc-tagged wild-type and N204Q mutant proteins were localized at the cell surface (Fig. 4, A and B). However, it appeared that N254Q and N337Q mutants were retained in the ER and showed clear co-localization with calnexin (Fig. 4, C and D). To further investigate whether Panx1 was localized at the cell surface, we biotinylated intact rPanx1-WT or rPanx1-Myc expressing HEK293T cells and analyzed the protein lysates by SDS-PAGE and Western blot. The biotinylated proteins were recovered by passage through an avidin resin column (bound fraction) and compared with the total protein lysate (unbound fraction, nonbiotinylated). As shown in Fig. 5, A and B, lanes 1 and 2, the upper band (GLY2) was biotinylated, demonstrating that it corresponds to the protein on the cell surface. Because the upper band was insensitive to Endo H treatment, we concluded it corresponds to mature-type glycoproteins (trans-Golgi glycosylated proteins). Although the intermediate band is found in the bound fraction, this is most likely due to some residual intracellular proteins labeled by the biotinylation reagent, as shown in the lower part of the blot hybridized with an antibody against α-tubulin. However, there is relatively little labeling of α-tubulin in the bound fraction when compared with the unbound one. Cell surface biotinylation of the N mutants (Fig. 5B, lanes 3–8) further confirmed the cell surface expression of the N204Q mutant only. To summa-

FIGURE 4. Co-localization of Myc-tagged wild-type and N mutant rPanx1 proteins with calnexin. Anti-Myc staining (left inset in black and white), calnexin staining (middle inset in black and white), and the merged view (right inset, yellow) are shown. A z-scan by confocal microscopy along the indicated axis (white dashed lines in magnified view) shows the N254Q and N337Q mutants co-localized with calnexin, indicating that the protein is retained in the ER. Scale bar, 10 μm.
Pannexin1 Multimeric Channels Are Glycosylated

Deglycosylation by Pharmacological Treatment Prevents the Expression of Panx1 on the Cell Surface. A, Western blot analysis of cellular lysates from MDCK cells stably expressing a tetracysteine domain-tagged rPanx1 (rPanx1-4C) before and after tunicamycin treatment (2 μg/ml) for the indicated time periods (h). The blots were hybridized with anti-Panx1 antibody, stripped, and re-probed against anti-α-tubulin as loading control. B, tunicamycin treatment over periods of time resulted in a significant decline in the fully glycosylated Panx1 proteins (GLY2), and an increase in the core proteins (GLY0). The relative densities of the different forms of protein (GLY0, non-glycosylated core protein, GLY1, the high mannose-type glycoprotein, and GLY2, the fully processed glycoprotein) were quantified and represent the mean ± S.E. of four independent Western blots.

Deglycosylation by Pharmacological Treatment Prevents the Expression of Pannexin1 on the Cell Surface—In addition to site-directed mutagenesis, we tested pharmacologically the effect of protein glycosylation in targeting Panx1 to the plasma membrane. We used tunicamycin, which inhibits transfer of the initial high mannose unit to the nascent peptide and thus prevents all N-linked glycosylation. MDCK cells stably expressing a tetracysteine domain-tagged rPanx1 (rPanx1-4C) were treated with tunicamycin (2 μg/ml) for different incubation times (Fig. 6). Over the periods of time tested, Western blot analysis revealed a significant decline in the fully processed Panx1 glycoproteins (GLY2), and an increase in the non-glycosylated core proteins (GLY0).

To spatially segregate nascent versus older Panx1 proteins we used the pulse-chase labeling protocol with green fluorescent FlAsH-EDT2, and red fluorescent ReAsH-EDT2 (16). This allowed us to label two temporally distinct pools of rPanx1-4C. MDCK cells stably expressing rPanx1-4C were stained with FlAsH-EDT2, incubated for different periods of time (chase time) in regular medium or in the presence of tunicamycin, then labeled with ReAsH-EDT2, fixed, and imaged (Fig. 7). Immunolabeling of the same cells with a polyclonal antibody for Panx1 confirmed the specificity of FlAsH/ReAsH staining (data not shown). The newer rPanx1-4C proteins were distributed throughout the plasma membrane mixing with the older ones. No punctate staining resembling the typical gap junction plaque was observed. As a control, the temporal order of FlAsH-EDT2, and ReAsH-EDT2 labeling was reversed, which produced a similar protein distribution. After 15–18 h the presence of rPanx1-4C initially labeled with the green fluorescent FlAsH-EDT2 was increased in cytoplasmic vesicles, which did not contain both colors suggesting the older proteins were targeted to this compartment for degradation. Following tunicamycin treatment the nascent Panx1-4C proteins accumulated within the cytoplasm and no labeling on the plasma membrane was observed. Furthermore, following treatment with the protein synthesis inhibitor, cycloheximide, there was no labeling in the intracellular or the plasma membrane compartment (data not shown). These data confirmed the importance of glycosylation in targeting the newly synthesized Panx1 proteins to the cell surface.

To further investigate the nature of the cytoplasmic vesicles containing the older proteins, rPanx1-4C were stained with ReAsH-EDT2, incubated for 15 h in regular medium, labeled with Lysotracker Blue, and then live imaged (Fig. 8). The merged view demonstrated the co-localization of Panx1 with lysosomes highlighting an important role of lysosomes in Panx1 degradation in this cell type.

EM Imaging of Pannexin1 Proteins—To test whether Panx1 proteins would form gap junction-like structures we imaged these proteins at high resolution EM using 4C technology and photoconversion (16) to specifically label rPanx1-4C or rCx43-GFP-4C (Fig. 9, A and E) stably expressed in MDCK cells. One of the advantages of this technology is the ability to perform correlated light and electron microscopy. The cells were labeled with ReAsH-EDT2, fixed, and then processed for photoconversion of diaminobenzidine. Photoexcitation of ReASH in the presence of oxygen generates singlet oxygen, which locally polymerizes diaminobenzidine to produce an electron dense reaction product allowing the tetracysteine-tagged proteins to be imaged at EM resolution. In Fig. 9, A and E, the images represent staining with ReAsH-EDT2, obtained in living cells before photoconversion; the electron micrographs of corresponding cells at higher magnification (Fig. 9, B, C, F, and G) show that Panx1 in areas of cell to cell apposition does not
appear as canonical gap junctions, and the intercellular spacing between adjacent cells is 20–50 nm compared with 2–5 nm in the Cx43-based gap junction. For the majority, Panx1 is dispersed across the plasma membrane (Fig. 9D), whereas Cx43 is not (Fig. 9H), but rather concentrates in plaques. As a control, cells outside the photoconverted area do not show any reaction product (Fig. 9I).

**Pannexin1 Does Not Form Functional Gap Junctions**—Functional studies were performed to assess whether Panx1 proteins were able to form intercellular channels. A classic assay for gap junction coupling is the scrape-load dye transfer assay (17). We performed these experiments in communication-deficient MDCK cells that endogenously expressed Panx1 or in MDCK cells stably expressing rPanx1-WT or a tetracysteine domain-tagged rPanx1 (rPanx1-4C). Immunolabeling was performed directly after fixation for post-dye coupling to verify protein expression. As shown in Fig. 10, transfer of Lucifer Yellow took place in MDCK cells expressing rCx43-4C, but not in MDCK expressing endogenous Panx1, or stably expressing rPanx1-WT or rPanx1-4C.

A more rigorous and quantitative assay for gap junction coupling is the delayed pairing paradigm in Xenopus oocytes (24). Oocytes were injected with Panx1 cRNA and after 2–3 days of incubation in OR2 solution, devitellinized and paired. Junctional conductance was determined by dual voltage clamp at 6 and 24 h after pairing. Uninjected oocytes served as negative control and rCx46-injected oocytes as positive control. Cx46 was chosen as positive control because, when expressed in oocytes, Cx46 forms both gap junction channels and patent connexons in the nonjunctional membrane. This allows for an indirect measure of the amount of Panx1 and Cx46 protein expression in the membrane by determining the nonjunctional membrane conductance. Fig. 11 shows that Cx46 formed gap junctions efficiently. Junctional con-
ductance was prominent already within 1 h after pairing and increased further with longer pairing periods. The junctional conductance in Panx1 expressing oocyte pairs, on the other hand, was at the detection threshold even 6 h after pairing (Fig. 11B). Panx1 failed to form a significant number of gap junction channels despite high expression levels as indicated by the Panx1-specific membrane currents (Table 1). Based on the measured membrane conductances and the unitary conductances of Cx46 and Panx1 channels, 70% of Cx46 were in the form of gap junction channels, whereas 95.3% of mPanx1 were in the form of unpaired pannexons.

The non-junctional conductances for Cx46 and Pannexin1 differed by a factor of 5 when equal concentrations of cRNA were injected (Table 1). Assuming similar gating phenomena, the protein pools in the plasma membrane therefore might differ by the same factor. Because the rate of gap junction channel formation is highly dependent of the precursor pool size (24), the observed differences in the ability to form gap junction channels in part could be attributed to this effect. To compare gap junction formation rate at similar pool sizes, the Cx46 cRNA was injected at a 1:20 dilution and the oocytes were paired 12 h after injection. The non-junctional conductances attributable to Cx46 as determined by voltage steps from −50 to 0 mV were 3.2 ± 0.4, 4.2 ± 0.4, and 5.2 ± 0.9 μS for time points 2, 6, and 24 h after pairing, respectively. Fig. 12 shows that the gap junction formation rates of Cx46 even under this condition by far exceeded that of the rate achieved by Panx1.

In similar experiments with *Xenopus* oocytes injected with the N254Q mutant, of 28 cells tested only 4 showed a detectable membrane conductance (1.7 ± 0.2 μS, mean ± S.E., n = 4) and also junctional conductance (0.5 ± 0.3 μS, mean ± S.E., n = 4) attributable to the
N254Q mutant expression and yielding a g/gm ratio of 0.3. The remaining oocytes exhibited a membrane conductance like un.injected oocytes (1.0 ± 0.1 µS, mean ± S.E., n = 24). This supports a mechanism of impaired trafficking due to a lack of glycosylation.

DISCUSSION

The data presented here show for the first time that Panx1 forms a multimeric channel that is N-glycosylated on the second extracellular loop at position Asn-254. Our results based on cross-linking experiments suggest that Panx1 forms a hexameric channel similarly to a connexin hemichannel; we observed several cross-linked products that based on molecular weight correspond to the monomer, dimer, and hexamer of rPanx1. Bands higher than the corresponding hexamer were not observed even at a higher concentration of DSP indicating that the chemical cross-linker allowed the formation of an oligomeric complex rather than an aggregate.

The addition and subsequent processing of carbohydrates to proteins occur in the lumen of the endoplasmic reticulum (ER) and in the Golgi in different steps. Results shown here demonstrate that rPanx1 is N-glycosylated and three different forms are observed: 1) the non-glycosylated core protein (GLY0) that is not affected by the glycosidase treatment; 2) the high mannose-type glycoprotein residing in the ER (GLY1) that is sensitive to Endo H treatment; and 3) the fully processed protein (GLY2, mature-type glycoprotein, trans-Golgi glycosylated) that is expressed on the cell surface. Glycosylation occurs only at sites located in the lumen of the ER that correspond to the extracellular domain of the protein. Our studies indicate that the Asn-254 is localized to the extracellular domain of the protein validating the predicted transmembrane topology of Panx1. In agreement with these observations, confocal immunofluorescence microscopy on cells expressing Panx1 proteins carrying a Myc tag at the carboxyl terminus demonstrated that the epitope was accessible to the antibody only when the cells had been permeabilized (data not shown). These results match the observations by Locovei and colleagues (3) in which an “extracellular loop” antibody applied to intact Xenopus oocytes gave intense staining on the cell surface, whereas the “carboxyl antibody” labeled oocytes only after cryosectioning.

Expression of three N mutants revealed that trafficking to the cell surface is disrupted by two of these three Asn → Gln mutations; however, Western blot analyses confirmed that only the N254Q mutant is entirely glycosylation-deficient, whereas N337Q is not. These results suggest that glycosylation at Asn-254 is important for plasma membrane targeting of the Panx1 protein. Furthermore, preventing N-glycosylation by pharmacological treatment resulted in an accumulation of the nascent Panx1 proteins within the cytoplasm with no labeling on the plasma membrane. Interestingly, the N337Q mutant was only partially glycosylated, as indicated by the presence of the intermediate (GLY1) band, and its trafficking to the cell surface was disrupted. We propose that Asn-337 may be relevant to the fully processed glycoprotein but possibly via interactions with chaperone-like proteins associated with the ER or Golgi, which may affect its final glycosylation process en route to the plasma membrane.

The ability to spatially segregate nascent versus older proteins is a powerful tool in understanding dynamics of the life FIGURE 10. Pannexin1 channels failed to transfer Lucifer Yellow in scrape-loading/dye transfer assays. A, representative immunofluorescence images of endogenous expression of Panx1 in MDCK cells, or stable cell lines expressing rPanx1-wt, rPanx1-4C, or Cx43-4C. B, all different cell types expressing Panx1 failed to transfer Lucifer Yellow in a scrape-loading dye transfer assay. C, MDCK cells expressing Cx43-4C successfully transfer Lucifer Yellow in a scrape-loading assay. D, summary of intercellular Lucifer Yellow diffusion reported as number of Lucifer Yellow positive cells observed for the different groups.
Pannexin1 Multimeric Channels Are Glycosylated

![Image](71x659 to 118x674)

![Image](71x682 to 118x713)

**FIGURE 11.** Junctional and non-junctional membrane currents in oocytes expressing Cx46 or Panx1. A, oocytes were injected with the respective cRNA for Cx46 (panel 1) and Panx1 (panels 2–4) and paired 3 days later. Membrane currents $i_1$ and $i_2$ induced by 5 (panel 1), 10 (panel 2), or 80 (panel 3) mV voltage steps were recorded 1 (panel 1) or 6 h (panels 2–4) after pairing. The membrane holding potential was 0 mV (panel 1) or −30 mV (panels 2–4). The calibration bar for the currents represents 500 nA for $i_1$ and 100 nA for $i_2$ for panels 1–3. In panel 4 the current traces of panel 2 are scaled up $\times 10$. The $i_1$ current is the sum of membrane current and junctional current, and at the 1-h time point for Cx46 this $i_1$ current is much larger than the junctional current ($i_j$ alone) (panel 1). For the Panx1 expressing oocyte pairs even at 6 h after pairing the junctional current is barely detectable, whereas the membrane current is large. B, junctional conductances of oocyte pairs expressing mouse or rat Pannexin1 (mPanx1, rPanx1) or rat connexin 46 (rCx46). Uninjectected oocytes served as control. Conductances were determined 6 (open bars) and 24 h (black bars) after pairing, Cx46 was injected 2–3 days before pairing. The junctional conductances of rCx46 expressing oocytes paired for 24 h were too large to be determined accurately.

**TABLE 1**

|                      | Control | mPanx1 | rPanx1 | rCx46 |
|----------------------|---------|--------|--------|-------|
| $g_{m}$ (µS)         | 1.3 ± 0.1 (19) | 6.8 ± 1.1 (13) | 6.4 ± 0.8 (12) | 26.9 ± 2.4 (9) |
| $g_{j}$ (µS)         | 0.02 0.07 | 0.17 ± 0.03 | 0.07 ± 0.01 | 31.4 ± 10.6 |
| $g_{m}$/$g_{j}$      | 1.3 0.01 | 0.17 0.07 | 1.3 1.01 | 26.9 31.4 |

Non-junctional and junctional conductances of oocyte pairs expressing Panx1 or Cx46.

Junctional and membrane conductances (in µS) were measured 6 h after pairing. Mean ± S.E. are given (number of cells or cell pairs).

 cycle of proteins. We used the pulse-chase labeling protocol with green fluorescent FlAsH-EDT2 and red fluorescent ReAsH-EDT2 to label two temporally distinct pools of rPanx1-4C. New and older proteins appeared to be mixed with each other at the plasma membrane. Older Panx1 proteins chased for 15–18 h were targeted to lysosomes suggesting the involvement of this proteolytic pathway for Panx1 degradation. No punctate staining resembling the typical gap junction plaque was observed as confirmed also by immunofluorescence of WT and Myc-tagged Panx1 proteins in native and recombinant systems. Furthermore, the EM resolution of the photoconverted Panx1 proteins confirmed their distribution on the cell surface. At apposed plasma membranes the intercellular spacing between adjacent cells was 20–50 nm. Interestingly, the addition of bulky carbohydrate moieties to the extracellular spacing of Panx1 would prevent the close apposition necessary to make a tight seal between two hemichannels therefore impeding the assembly into a gap junction. Indeed, insertion of patent glycosylation sites into connexins rendered these gap junction-forming proteins non-functional (25). Furthermore, simple dye transfer assays show that these oligomers do not pass Lucifer Yellow dye between cells as has been shown for connexins.

The presence of a glycosylation site at the extracellular surface, the distribution on the plasma membrane of Panx1, and the lack of dye transfer argue that pannexins form a new class of channel proteins distinct from connexins, confirming earlier studies in erythrocytes by Dahl and co-workers (3) and in HeLa cells, astrocytes, and neurons by Huang and colleagues (4). A recent paper by Lai et al. (26) showed increased dye coupling in C6 glioma cells upon transfection with exogenous Panx1-myc or Panx1-EGFP. These cancer cells do not express connexins or pannexins; however, astrocytes from which these cells are derived do express connexins and pannexins. Because exogenously transfected Panx1-EGFP fluorescence occurred all over the plasma membrane, it is possible that the restored Panx1 induced up-regulation of connexins and therefore, cells became coupled through connexin intercellular channels rather than putative Panx1-EGFP intercellular channels.

In paired oocytes, Panx1 expression resulted in the formation of junctional conductance only with excessive cell to cell contact times of 24 h and more. This finding is in agreement with the original report by Bruzzone et al. (2) where 24–48 h pairing periods were used to assess the ability of Panx1 to form gap junction channels.

Under more stringent recording conditions, i.e. measurement of junctional conductance shortly after pairing from an accumulated pool of precursors, no significant gap junction
function attributable to Panx1 could be detected within a 6-h period after establishment of cell to cell contact. However, Panx1 was responsible for a large voltage-dependent non-junctional membrane conductance indicating high expression levels of the protein. In contrast, Cx46 produced gap junction channels very efficiently. A large junctional conductance was present already 1 h after pairing and within 6 h after pairing about 210,000 open gap junction channels and ~90,000 non-junctional open connexons were counted. Given the dynamic nature of cells and their interactions the requirement for extended contact periods in the order of days for Panx1 to form gap junction channels appears incompatible with a physiological role in a native system except in cells in non-regenerating tissues where cell-cell interactions might be more stable.

In the oocyte expression system, the non-glycosylated form of Panx1 represents a substantial fraction of the protein (3). Also, glycosylation in these cells is faulty (27–29). It is conceivable that despite the trafficking defect some unglycosylated Panx1 reaches the plasma membrane and can be used for gap junction channel formation. Although this process may be inefficient, an atypically long cell-cell contact period facilitates detection of such a rare event. Furthermore, the forced large contact area in oocyte pairs can be an enabler of an otherwise improbable event. Therefore, the observation of gap junction formation in paired oocytes (2) could be a peculiarity of the expression system based on faulty glycosylation. The formation of gap junction channels in this system may not reflect the in vivo ability of the pannexin protein.

In conclusion, our study shows that rPanx1 cell surface expression can be altered by multiple factors such as a sugar tree addition and a COOH-terminal motif. Pannexins are expressed in many different tissues, and the cell surface expression levels vary in different locations. In astrocytes and endothelial cells, for example, the expression pattern of Panx1 appears to be mainly intracellular, and only the lower band corresponding to the non-glycosylated core protein is observed by Western blot. Glycosylation of membrane proteins can affect their folding, stability, trafficking, and function. We propose that N-glycosylation of Panx1 could be a significant mechanism for regulating the trafficking of these membrane proteins to the cell surface and possibly their function in different tissues.

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