Pathways Regulating the Trafficking and Turnover of Pannexin1 Protein and the Role of the C-terminal Domain*

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Pannexins were originally discovered due to their shared sequence homology with the invertebrate gap junction proteins, innexins (1). The pannexin family is comprised of three members as follows: Panx1, Panx2, and Panx3 (1). Although the scope of physiological significance of pannexin family members is only beginning to emerge, it has been reported that Panx1 holds importance in forming conduits for ATP release (2–4), propagation of Ca\(^{2+}\) waves (5), and neuronal and immunological inflammasome (6–8). Additionally, activation of Panx1 channels has been implicated in ischemic cell death (9) and seizure-like activities (10). Although Panx1 single channel conductances of 550 picoamperes (2) have been correlated with ionic dysregulation during ischemic conditions, subsequently leading to neuronal necrosis (9), the N-methyl-D-aspartate receptor-induced opening of Panx1 channels has been implicated in hippocampal epileptiform seizure-like activity (10).

Other studies have associated Panx1 channel activation with apoptosis of Xenopus oocytes by forming a pore unit with the death complex of the P2X7 receptor (11), whereas Qu et al. (12) and Chekeni et al. (13) have discovered the use of Panx1 channels for ATP release during apoptosis. In light of the broad physiological relevance of Panx1 channels, it is critical to determine the pathways and mechanisms that regulate their assembly and turnover.

We and others have shown that Panx1 is N-glycosylated (14, 15) and exists as core (Gly0), high mannose (Gly1), and complex (Gly2) species (16). The significance of Panx1 glycosylation on trafficking was first explored using site-directed mutagenesis of N-linked glycosylation sites (14). The Panx1\(^{N254Q}\) glycosylation-deficient mutant displayed trafficking defects suggesting that glycosylation plays a role in proper trafficking of Panx1 to the cell surface. In Madin-Darby canine kidney cells, Panx1 has been shown to rescue the delivery of the Panx1\(^{N254Q}\) mutant to the cell surface suggesting that they co-oligomerize (17). Thus, the lack of glycosylation does not perturb the ability of Panx1\(^{N254Q}\) subunits to fold properly and intermix with Panx1. Interestingly, despite the glycosylation deficiency and the low levels of the Panx1\(^{N254Q}\) mutant at the cell surface, the mutant was still capable of forming functional channels suitable for dye uptake (15). A glycosylation-deficient mutant of zebrafish Panx1 (zfPanx1-N246K), however, reached the cell surface but displayed impaired uptake of ethidium bromide compared with wild type zfPanx1 (18).

The function of Panx1 channels has also been investigated by engineering specific point mutations in the polypeptide sequence and expressing these mutants in reference cell models. Substitution of lysines to alanines, for instance, at residues 248 or 265 in the second extracellular loop resulted in a loss of Panx1 channel function, and substitution of arginine at position 75 (in the first extracellular loop) to alanine, lysine, glutamate, or cysteine was further used to assess the inhibitory effects of ATP analogues on Panx1 channel currents (19). When expressed in Xenopus oocytes, mutation of a cysteine to serine

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at residue 346 located within the C-terminal tail resulted in constitutively leaky channels thereby causing cell death (20).

Although several studies have looked at the effect of single amino acid substitutions within specific Panx1 motifs, the deletion of the entire Panx1 domains has not been studied. In this study, we were particularly interested in understanding the role of the Panx1 C terminus by investigating the trafficking and oligomeric potential of C-terminal truncated Panx1. We chose the 127-amino acid long C-terminal domain as this cytoplasmic exposed domain has previously been shown to bind actin, which provides a multifaceted role in vesicular transport, cell surface mobility, and stability of Panx1 (21). More recently, the C terminus of Panx1 was characterized as a target for caspase-3 and -7 cleavage, which resulted in a constitutively open channel (13). In the same study, Panx1 channels were shown to mediate phagocyte recruitment by releasing nucleotide signals from apoptotic cells (13).

We and others have reported that Panx1 has a long half-life (15, 17), and one line of evidence supports the notion that internalized Panx1 is destined for lysosomal degradation (14). However, which endocytic pathway (24–30) governs Panx1 internalization remains to be investigated. Thus, the focus of this study was also to determine whether Panx1 employs classical endocytic pathways such as clathrin and/or caveolin for its internalization.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—BICR-M1R<sub>α</sub> cells, originally derived from rat mammary tumors, were a gift from Dieter Hulser, Stuttgart, Germany (22), and were engineered to stably overexpress Panx1, as described previously (15). Both BICR-M1R<sub>α</sub> and human embryonic kidney (HEK)-293T cells were engineered pEGFP-N1 vector (15) using HindIII and BamHI restriction enzymes and inserted into the pcDNA3-mRFP vector (Addgene Plasmid Repository). Truncated Panx1 and RFP<sup>2</sup> (denoted Panx1<sup>T307-RFP</sup>) were separated by a five-amino acid polylinker encoded by the nucleotide sequence CTGTCGACGATACGGCGGAGGATCCACTAGTACGGCGGAGTGTGTGGAAATTCCTGCAGATATCCACTGTCGGCCGGCTCGAG.

For transfection of Panx1<sup>T307-RFP</sup> and Panx1-RFP, cells were grown overnight to 50–70% confluency in 35-mm dishes and transfected in Opti-MEM<sub>1</sub> media containing 2 μl of Lipofectamine 2000 (Invitrogen) and 5 μg of plasmid DNA. For co-expression studies, HEK-293T cells were plated in 100-mm dishes, and 7–9 μg of Panx1 were co-transfected with 10–15 μg of either Panx1<sup>T307-RFP</sup> or Panx1-RFP in Opti-MEM<sub>1</sub> media containing 15 μl of Lipofectamine 2000.

GFP-tagged WT and K44A DynII expression constructs were kindly provided by Dr. Mark A McNiven (Mayo Clinic College of Medicine, MN) and used for conducting transient transfections of BICR-M1R<sub>α</sub> cells expressing Panx1. Cells grown in 35-mm dishes overnight to ~50–70% confluency were transfected in Opti-MEM<sub>1</sub> media containing 1 μg of WT or K44A DynII GTPase cDNA constructs with 2 μl of Lipofectamine 2000. Opti-MEM<sub>1</sub> medium was replaced with complete culture media 4 h after transfection at 37 °C.

Immunocytochemistry—Cells were immunolabeled as described previously (21). Briefly, cells grown on glass coverslips were fixed using ice-cold 80% methanol and 20% acetone for 20 min at 4 °C and blocked in 2% blocking solution (bovine serum albumin (BSA; Sigma) in PBS) for 30 min. Cells were incubated for 1 h at room temperature with affinity-purified polyclonal Panx1 antibody at a concentration of 2 μg/ml, a 100-fold dilution of monoclonal anti-GM130 antibody (BD Transduction Laboratories), a 500-fold dilution of monoclonal anti-PDI antibody (StressGen), or a 200-fold dilution of polyclonal anti-RFP antibody (Abcam). Cells were incubated with goat anti-rabbit or goat anti-mouse Alexa Fluor488 (1:500, Invitrogen) for 45 min at room temperature. Cells were rinsed with PBS, and nuclei were stained with Hoechst 33342 and mounted.

Panx1 expressing BICR-M1R<sub>α</sub> cells fixed with 3.7% formaldehyde were permeabilized in PBS containing 1% BSA and 0.1% Triton X-100. Because antibodies against Panx1, clathrin, and Cav-1 were all raised in rabbits, an Alexa Fluor488-conjugated Panx1 antibody was synthesized as per the manufacturer’s directions (Pierce) for dual labeling of Panx1 with these markers. For immunolabeling of Panx1-expressing cells for clathrin or Cav-1, cells were first incubated with either a 200-fold dilution of polyclonal anti-clathrin heavy chain antibody (Abcam) or a 100-fold dilution of polyclonal anti-Cav-1 antibody (BD Transduction Laboratories) for 1 h at room temperature, followed by incubation with goat anti-rabbit Alexa Fluor555 (1:500, Invitrogen) for 45 min at room temperature. Thereafter, cells were washed with PBS and incubated with Panx1<sub>1488</sub> antibody for an additional 45 min at room temperature before staining nuclei with Hoechst 33342 (Invitrogen). For double labeling of Panx1-expressing cells for AP2 or Cav-2, cells were first labeled with affinity-purified polyclonal Panx1 antibody at a concentration of 2 μg/ml for 1 h, followed by incubation with goat anti-rabbit Alexa Fluor555 (1:500, Invitrogen) for 45 min at room temperature. Next, cells were labeled with either monoclonal anti-AP2 (Sigma) or monoclonal anti-Cav-2 (BD Transduction Laboratories) at a 100-fold dilution followed by

<sup>2</sup>The abbreviations used are: RFP, red fluorescent protein; DynII, dynamin II; ER, endoplasmic reticulum; MJ<sub>B</sub>, methyl-β-cyclodextrin; BFA, brefeldin A; PDI, protein-disulfide isomerase; Endo, endo-β-N-acetylglucosaminidase.
incubation with goat anti-mouse Alexa Fluor488 (1:500, Invitrogen). DynII was labeled using a 50-fold dilution of anti-DynII antibody (Santa Cruz Biotechnology) for 1 h prior to using Texas Red-conjugated donkey anti-goat (1:200, Jackson ImmunoResearch) as a secondary antibody. Immunolabeled cells were imaged using a 63 oil objective lens mounted on a Zeiss LSM 510 META system (Zeiss, Toronto, Ontario, Canada).

Pharmacological Inhibitors—Brefeldin A (BFA), dynasore, methyl-β-cyclodextrin (MβC), lactacystin, and chloroquine were all purchased from Sigma. BICR-M1R<sub>e</sub> and HEK-293T cells expressing Panx1<sup>T307</sup>-RFP and Panx1 were exposed to 10 μM lactacystin and 200 μM chloroquine for 20 h prior to protein extraction. Cells were treated for 20–32 h with 5 μg/ml BFA and for 1–24 h with 10 mM MβC. Dynasore treatment was used at a concentration of 40 μM, consistent with previous studies (23). Post-treatment, cells were fixed for immunocytochemistry or lysed for immunoblotting and co-immunoprecipitation assays.

Co-immunoprecipitation and Immunoblotting—Co-immunoprecipitation and immunoblotting assays were performed

**FIGURE 1. Truncated Panx1 fails to reach the cell surface.** When expressed in BICR-M1R<sub>e</sub> cells, Panx1<sup>T307</sup>-RFP was localized to intracellular compartments (A, red), and Panx1-RFP was found at the cell surface (B, red). Immunolabeling with anti-RFP antibody detected both Panx1-RFP and Panx1<sup>T307</sup>-RFP (A and B, green). Furthermore, protein lysates from HEK-293T WT cells and cells overexpressing Panx1-GFP, Panx1-RFP, or Panx1<sup>T307</sup>-RFP were immunoblotted with either anti-RFP antibody (C) or anti-Panx1 antibody (D). Panx1<sup>T307</sup>-RFP was detected primarily as a doublet of ~50 kDa (C), and Panx1-RFP (C and D) was resolved similar to Panx1-GFP (D) in a multiple banding profile above ~75 kDa. GAPDH was used as a protein loading control. Protein lysates of HEK-293T cells labeled with (+) or without (−) biotin were precipitated with NeutrAvidin beads prior to immunoblotting for RFP and Panx1. Cell surface biotinylation of Panx1-RFP revealed that all glycosylated species of Panx1 trafficked to the cell surface in the NeutrAvidin pulled samples (F), and no clear cell surface expression of Panx1<sup>T307</sup>-RFP was detected (E). GAPDH was used as a control to detect any internalization of biotin during the labeling procedure.
based on our previously described protocol (21). Briefly, cells were lysed in buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Nonidet P-40, and 1% Triton X-100 and supplemented with protease inhibitor mixture (1 tablet per 10 ml of buffer, Roche Applied Science) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). Protein concentrations were measured using a BCA protein determination kit (Pierce), and 20–30 μg of total protein lysate was resolved on a 10% SDS-polyacrylamide gel. Protein samples were transferred onto nitrocellulose membranes using the iBlot Dry Blotting system (Invitrogen) and blocked with 5% Blotto milk (Santa Cruz Biotechnology) or 3% BSA (Sigma) for 1 h prior to incubating with anti-Panx1 (0.2 μg/ml), anti-RFP (1:2000, Abcam), anti-clathrin (1:1000, Abcam), anti-AP2 (1:500, Sigma), anti-DynII (1:250, Santa Cruz Biotechnology), anti-Cav-1 (1:2000) or anti-Cav-2 (1:2000 BD Transduction Laboratories), and anti-GAPDH (1:5000, Millipore) antibodies overnight at 4 °C. Primary antibody binding was detected using mouse IgG infrared dye 800 at 1:10,000 dilution (Rockland Immunochemicals) or rabbit IgG Alexa Fluor680 at 1:10,000 dilution (Invitrogen), and blots were scannned with the Odyssey infrared imaging system (Li-Cor).

Co-immunoprecipitation assays were performed as described previously (24). Briefly, 800–1000 μg of cell lysates were incubated with 10 μg/ml anti-Panx1 and 2 μg/ml specific anti-Cav-1 or anti-Cav-2 antibodies (BD Biosciences) antibodies and rocked overnight at 4 °C. The following day, 30 μl (50% slurry) of protein A-Sepharose beads were added to the lysates and incubated for an additional 2 h at 4 °C. Thereafter, beads were collected by centrifugation at 4500 rpm for 2 min and washed three times to remove any nonspecific binding. The samples were boiled for 5 min in 2× Laemmli buffer prior to resolving on 8–10% SDS-polyacrylamide gels and immunoblotting with specific antibodies to detect Panx1, RFP, clathrin, AP2, Cav-1, Cav-2, and DynII at concentrations mentioned above. Although primary antibodies were detected using mouse IgG IR dye 800 at 1:10,000 (Rockland Immunochemicals) or rabbit IgG Alexa Fluor680 at 1:10,000 (Invitrogen), detection of co-immunoprecipitates was also achieved using the Clean-Blot IP detection reagent (horseradish peroxidase;
Pierce) as a secondary antibody at a 4000-fold dilution for 45 min to minimize the appearance of the IgG bands in some instances.

Deglycosylation Assays—Enzymatic digestion of glycan chains using peptide N-glycosidase F and endo-β-N-acetylglucosaminidase (Endo) H was performed as described previously.
Briefly, 35 μg of HEK-293T cell lysates containing either Panx1T307-RFP or Panx1-RFP were incubated in the presence or absence of 10 units of N-glycosidase F and incubated for 1 h at 37 °C prior to immunoblotting using the RFP antibody. Similarly, for Endo H treatment, 35 μg of protein was incubated with or without 5000 units of the enzyme for 1 h at 37 °C, and samples were resolved on a 8% SDS-PAGE before transferring to nitrocellulose membranes and probing with an anti-RFP antibody.

Cell Surface Biotinylation—HEK-293T cells ectopically expressing either Panx1 alone or with Panx1-RFP and Panx1T307-RFP were subjected to biotinylation 48 h following transfection. To prevent any potential internalization of cell surface proteins, all reagents and cell cultures were maintained on ice. Cell culture dishes were first rinsed three times with cold PBS to remove media and incubated thereafter either in PBS alone or in PBS containing 1 mg/ml of EZ-link Sulfo NHS-LC biotin (Pierce) for 20 min at 4 °C. To quench any excess biotin, cell culture dishes were first rinsed and then incubated in PBS containing 100 mM glycine for an additional 15 min at 4 °C. Proteins were then extracted from both unlabeled and biotin-labeled dishes using the SDS lysis buffer (1% Triton X-100 and 0.1% SDS in PBS) and quantified using a BCA protein determination kit (Pierce). 1 mg of cell lysates from biotin-labeled and unlabeled samples was incubated with 50 μl of NeutrAvidin-agarose beads (Pierce) overnight at 4 °C. The following day, beads were washed with IP lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Nonidet P-40, and 1% Triton X-100) containing 1 mM NaF and 1 mM Na3VO4 and suspended in 2× Laemmli buffer before boiling for 5 min and resolving on the SDS-PAGE. Total protein containing 30 – 40 μg of lysate was simultaneously resolved on the gel, transferred to nitrocellulose, and probed for RFP and Panx1 expression.

RESULTS

To characterize the biological significance of the Panx1 C-tail, we truncated Panx1 at residue 307, which is ~10 amino acids downstream of where the fourth transmembrane domain

![Image of Panx1 Trafficking, Internalization, and Degradation](image-url)
is predicted to exit the plasma membrane. To facilitate detection of the Panx1-truncated mutant, the mutant was tagged with RFP. To validate that the RFP tag does not modify the trafficking of Panx1, we also tagged full-length Panx1 with RFP and compared it with the untagged and GFP-tagged Panx1. Because RFP-tagged Panx1 exhibited a similar trafficking and distribution profile as the untagged and GFP-tagged counterparts (supplemental Fig. 1), we employed it as a control to study the biological properties of Panx1T307-RFP.

**C-tail of Panx1 Is Necessary for Panx1 Trafficking to the Plasma Membrane**—Both Panx1T307-RFP and Panx1-RFP were overexpressed in either BICR-M1Rk or HEK-293T cells, as these cell lines are devoid of endogenous Panx1 (16, 21). The high transfection efficiency of HEK-293T cells was deemed ideal for immunoblotting experiments; however, given their small spindle morphology, large nuclei, and low cytoplasmic volume (16), they were not as suitable for immunolocalization studies as the larger BICR-M1Rk cells (supplemental Fig. 2).

When Panx1T307-RFP was expressed in BICR-M1Rk cells, the mutant was found to primarily reside in intracellular compartments (Fig. 1A). In contrast, full-length Panx1-RFP was capable of trafficking and localizing to the cell surface in a relatively uniform manner (Fig. 1B).

As a glycoprotein, it is well documented that Panx1 exhibits multiple banding patterns reflecting the core unglycosylated protein (Gly0), a high mannose-glycosylated species associated within the ER (Gly1), and the extensively glycosylated species that is modified in the Golgi apparatus prior to cell surface delivery (Gly2) (14–16). To address if Panx1T307-RFP also resolves as multiple species, lysates of HEK-293T wild type cells, cells overexpressing full-length Panx1 fusion proteins, and the C-tail truncated protein were subjected to immunoblotting using anti-RFP and Panx1 antibodies. Because of the fusion of RFP (14–16), C-tail truncated Panx1 was detected as a doublet at a molecular mass of ~50 kDa, whereas full-length Panx1 resolved as multiple bands at ~75–80 kDa (Fig. 1C). Consistent with our immunolabeling data (supplemental Fig. 2), the Panx1 CT-395 antibody (from here on denoted as Panx1 antibody) did not detect Panx1T307-RFP but identified full-length GFP- and RFP-tagged Panx1 (~75–80 kDa), as well as occasionally some bands near 50 kDa, which are likely proteolytic products (Fig. 1D). As expected, WT cells did not express any Panx1 (Fig. 1, C and D); however, some faint nonspecific bands were observed above 50 kDa when probed with the RFP antibody (Fig. 1C).

To determine the cell surface delivery status of the C-tail mutant of Panx1, we conducted a cell surface biotinylation assay on live HEK-293T cells expressing Panx1T307-RFP or Panx1-RFP. As depicted by Panx1 immunoblotting of NeutrAvidin pulled down fractions and cell lysates, all species of Panx1-RFP were capable of trafficking to the cell surface, with a preference for the Gly2 species (Fig. 1F). In contrast, despite detectable Panx1T307-RFP expression in the cell lysates, NeutrAvidin fractions contained virtually no Panx1T307-RFP, when probed with anti-RFP antibody (Fig. 1E). As a control, the lack of immunolabeling of GAPDH suggests that biotin did not penetrate into the cell. Collectively, these findings suggest that the C terminus of Panx1 is critical for delivery and retention of Panx1 at the cell surface.
Panx1T307-RFP Is Primarily Retained within the ER Compartment and Glycosylated to a High Mannose Form—To identify the subcellular localization of Panx1T307-RFP, cells were immunolabeled for ER (PDI and calnexin) and Golgi (GM130) resident proteins. Although the distribution patterns of both Panx1T307-RFP and PDI (an ER lumen protein) partially overlap, their localization patterns were not identical (Fig. 2A). In contrast, the ER integral membrane protein marker, calnexin, revealed strong co-localization with Panx1T307-RFP, particularly around the perinuclear region (Fig. 2B). Not surprisingly, Panx1-RFP at the cell surface did not localize with either PDI or calnexin (Fig. 2A and B). Additionally, labeling with the cis-Golgi matrix protein, GM130, revealed a distinct distribution profile from both Panx1T307-RFP and Panx1-RFP (Fig. 2C). Thus, in the absence of the C-tail it appears that Panx1 is largely retained in the ER compartment.

To determine whether the banding pattern observed for Panx1T307-RFP (Fig. 1C) was a result of glycosylation, we enzymatically digested the cell lysates using N-glycosidase F prior to immunoblotting. Within an hour, N-glycosidase F treatment of Panx1-RFP removed glycan chains associated with the Gly1 and Gly2 species and shifted the banding pattern to the level of Gly0 (Fig. 2D). Similarly, the upper band of Panx1T307-RFP, likely the Gly1 species, also shifted to the lower band (Gly0) upon N-glycosidase F digestion (Fig. 2E). To further assess the extent of glycosylation, both Panx1T307-RFP and Panx1-RFP were subject to Endo H digestion, which selectively cleaves the high mannose form of a glycoprotein. Consistent with previously reported digestion of mouse (14, 16) and rat untagged Panx1 (14), Endo H treatment of Panx1-RFP revealed a shift in only the intermediate Gly1 band without reducing the Gly2 species (Fig. 2F). Interestingly, the upper band of Panx1T307-RFP was also sensitive to the Endo H treatment (Fig. 2G). Taken together, these results suggest that in the absence of the C-tail, glycosylation of Panx1 is limited to a high mannose form.

Panx1T307-RFP Acts as a Dominant-negative on the Maturation of Panx1 to the Gly2 Species and Its Delivery to the Cell Surface—To examine whether the C-tail of Panx1 regulates homomeric interactions, we transiently co-expressed Panx1 with Panx1T307-RFP. When Panx1 was ectopically expressed alone or in conjunction with Panx1-RFP, a relatively uniform cell surface distribution was revealed (Fig. 3, A and B). In contrast, the intracellularly retained Panx1T307-RFP was not rescued to the cell surface when co-expressed with Panx1; instead, an intracellu-
FIGURE 7. Panx1 co-distributes but does not co-immunoprecipitate with clathrin or AP2. Panx1 expressing BICR-M1Rk cells were immunolabeled for clathrin heavy chain (A) or anti-AP2 (B and C). Alexa Fluor488-conjugated Panx1 antibody detected Panx1 (green) with clathrin (red) at the cell surface (A, merge). Double immunofluorescent labeling revealed the co-distribution of Panx1 (green) with AP2 (red) at the cell surface before and after a 20-h BFA exposure (C). Nuclei were stained with Hoechst. Bar, 10 μm. Immunoprecipitates of Panx1 from WT and Panx1-expressing cells were immunoblotted for Panx1, clathrin, and AP2. Panx1 (43–50 kDa) was successfully pulled down from Panx1-expressing cells; however, clathrin (~192 kDa) or AP2 (~105–110 kDa) did not co-immunoprecipitate with Panx1 (D). IB, immunoblotting; IP, immunoprecipitation.
lular subpopulation of Panx1 was detected in the perinuclear region (Fig. 3C). Thus, the intermixing of Panx1 with the truncated mutant does not result in the rescue of the mutant to the cell surface.

To determine the effect of the Panx1T307-RFP mutant on the delivery of Panx1 to the cell surface, we conducted a cell surface biotinylation assay on live HEK-293T cells expressing Panx1T307-RFP and Panx1-RFP alone or in combination with Panx1. When co-expressed with Panx1, a subpopulation of the highly glycosylated species of Panx1-RFP (Fig. 3, D and E), as well as all three glycosylation species of Panx1, reached the cell surface (Fig. 3E). However, when co-expressed with Panx1T307-RFP, there was a noticeable reduction in the ability of all three species of Panx1 to reach the cell surface (Fig. 3E). Additionally, there was no strong evidence of plasma membrane localized Panx1T307-RFP, even in the presence of Panx1 (Fig. 3D). These findings support the notion that the Panx1T307-RFP mutant is acting dominantly to reduce the delivery of Panx1 to the cell surface.

C-tail of Panx1 Regulates the Oligomerization Efficiency of Panx1—To examine the potential role of the C-tail domain in Panx1 oligomerization, lysates of HEK-293T cells and cell over-expressing Panx1 alone or in combination with either Panx1-RFP or Panx1T307-RFP were subjected to immunoprecipitation for Panx1. When immunoblotted with either RFP or Panx1 antibodies, immunoprecipitates of Panx1 co-expressed with Panx1-RFP revealed both the high mannose (Gly1) and complex glycosylated species (Gly2) with much lower detection of the core (Gly0) species (Fig. 4, A and B). In contrast, only trace amounts of the Gly1 species of Panx1T307-RFP were found to co-immunoprecipitate with Panx1 (Fig. 4A). Reciprocally, immunoblots with the anti-Panx1 antibody revealed the Gly1 and Gly0 species with little evidence of the Panx1 Gly2 species when co-expressed with Panx1T307-RFP (Fig. 4B). Interestingly, total cell lysates also revealed little Gly2 species of Panx1 in the presence of Panx1T307-RFP (Fig. 4B). Collectively, these findings suggest that all three species of Panx1 can readily interact with Panx1-RFP; however, when the C-tail domain of Panx1 is removed, a weak interaction exists with Panx1, with a preference for the core and high mannose species.

Unlike Full-length Panx1, Panx1T307-RFP Is Preferentially Degraded by Proteasomes—Given the predominant ER localization of Panx1T307-RFP, we assessed whether Panx1T307-RFP is targeted to proteosomal or lysosomal degradation using the pharmacological inhibitors lactacystin and chloroquine, respectively. Lactacystin treatment clearly showed a robust accumulation of both the Gly1 and Gly0 species of Panx1T307-RFP, with no detectable change in protein levels upon chloroquine exposure (Fig. 5A). On the contrary, inhibition of lysosomal degradation with chloroquine and not proteasomes revealed a pronounced increase in the Gly1 and Gly2 species of Panx1-RFP (Fig. 5B). When compared with Panx1T307-RFP alone (Fig. 5A), co-expression with Panx1 revealed an accumulation of the Gly1 species of Panx1T307-RFP upon chloroquine treatment, without considerably altering the expression upon lactacystin treatment (Fig. 5C). Furthermore, co-expression of Panx1T307-RFP with Panx1 showed an overall decrease in the Gly2 species of Panx1, with a noticeable reduction in the Gly1 species, upon chloroquine treatment (Fig. 6A). Interestingly, when expressed alone, Panx1 revealed a sustained expression of the Gly2 species, with a pronounced accumulation of the Gly1, upon chloroquine treatment (Fig. 6B). The subcellular distribution of Panx1 in the presence of chloroquine paralleled its banding profile as a perinuclear subpopulation was noticed (presumably lysosomes (14)), along with a cell surface distribution profile (Fig. 6C). As reported previously (21), BFA treatment reduced the Gly2 species of Panx1 with a subsequent increase in the Gly1 form (Fig. 6B), which was consistent with the loss of the cell surface distribution coinciding with an accumulation in an intracellular compartment (Fig. 6C). Inhibition of the lysosomal pathway in the presence of BFA resulted in slightly increased expression of the Panx1 Gly2 species when compared with BFA treatment alone (Fig. 6B). This finding suggests that accumulation of the existing cell surface pool of Panx1 is a result of blocking its internalization and degradation through the lysosomal pathway.

Panx1 Co-distributes but Does Not Co-immunoprecipitate with Clathrin or AP2—To explore the internalization mechanism of Panx1, we first targeted the clathrin-mediated endocytic pathway, as it is a well documented process that is initiated by the recruitment of clathrin and adaptors (such as AP2) to the plasma membrane before forming a coat around the endocytic vesicles and budding off (25). We first immunolabeled Panx1 expressing BICR-M1RK cells with antibodies directed against the clathrin heavy chain and Panx1. Panx1 co-distributed with clathrin (Fig. 7A) and AP2 (Fig. 7B) at the cell surface. The plasma membrane co-distribution of Panx1 with AP2 was not noticeably altered in the presence or absence of BFA (Fig. 7, B and C). Additionally, immunoprecipitation of Panx1 from WT and Panx1-overexpressing cells did not co-immunoprecipitate protein complexes that contained either clathrin or AP2, which were expressed in total lysates at ~180 and ~105–110 kDa, respectively (Fig. 7D). As expected, Panx1 was immunoprecipitated in cells overexpressing Panx1 but not in WT cells (Fig. 7D). These studies suggest that Panx1 does not reside in protein complexes that contain constituent molecules of the clathrin-mediated endocytic pathway.

**FIGURE 8.** Cell surface Panx1 is localized to a distinct compartment and does not co-immunoprecipitate with Cav-1 or Cav-1. BICR-M1RK, cells endogenously expressing Cav-1 and Cav-2 and stably expressing Panx1 were fixed with formaldehyde or acetone/methanol prior to immunolabeling. Panx1 (green) was found to co-distribute with intracellular and plasma membrane-localized Cav-1 and Cav-2 (red) (A–D). Immunoprecipitates of Panx1 (E and G) from WT and Panx1-expressing cells were immunoblotted for Panx1, Cav-1, and Cav-2. Panx1 was detected only in the immunoprecipitates of Panx1 (E and G) and not in the immunoprecipitates of Cav-1 (F) or Cav-2 (H). Conversely, Cav-1 (F) and Cav-2 (H) were found in their respective immunoprecipitates and not in the Panx1 immunoprecipitates (E and G). Treatment of Panx1-expressing cells up to 24 h of M6C did not alter the expression levels of the Panx1 glycosylation species (I); the highly phosphorylated form of CX43 was reduced within 1 h of M6C exposure (I). The cell surface distribution of Panx1 (red) remained relatively uniform during the 24 h of M6C treatment (J). Nuclei were counterstained with Hoechst. Bar, 10 μm. GAPDH was used as a protein loading control. IB, immunoblot; IP, immunoprecipitation.
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Panx1 Co-distributes but Does Not Co-immunoprecipitate with Cav-1 or Cav-2—Cav-1 is ubiquitously expressed in several tissues, whereas Cav-2 is co-expressed with Cav-1 in various tissues, and Cav-3 is primarily restricted to muscle (26). To determine whether caveolins play a role in Panx1 trafficking or internalization, we investigated the potential relationships between Panx1 and Cav-1 or Cav-2. Formaldehyde and acetone/methanol fixatives were employed to detect the intracellular and cell surface populations of Cav-1/Cav-2, respectively (24). In formaldehyde-fixed cells, Panx1 co-distributed with Cav-1 (Fig. 8A) and Cav-2 (Fig. 8C) in the intracellular compartment, presumably the Golgi complex. Conversely, fixation with acetone/methanol revealed the co-distribution of Panx1 with Cav-1 (Fig. 8B) and Cav-2 (Fig. 8D) mainly at sites of cell-cell apposition. To examine the potential interaction of Panx1 with protein complexes that included Cav-1 and Cav-2, co-immunoprecipitation assays were conducted. As shown in Fig. 8, E–H, reciprocal co-immunoprecipitations revealed that Panx1 and Cav-1/Cav-2 were not part of the same protein complexes.

Although caveolins become detergent-insoluble upon integrating into membrane rafts, these rafts can be disrupted with the cholesterol-depleting agent MβC (24). Exposure of Panx1-expressing cells to MβC for up to 24 h to disrupt caveolin-enriched membrane rafts revealed no substantial difference in the Panx1 banding pattern (Fig. 8I) or localization profile (Fig. 8J). As a control to ensure lipid rafts were disrupted, the highly phosphorylated species of Cx43, typically associated with the cell surface and detergent-insoluble pool (24), diminished within 1 h of MβC treatment (Fig. 8I). Overall, our results indicate that Panx1 is not associated with caveolins or cholesterol-enriched membrane rafts.

**Panx1 Internalization Is Independent of Dynamin GTPase**—A dominant-negative GTP-hydrolyzing mutant of dynamin II (K44A) inhibits the function of endogenous dynamin (27); therefore, we transiently transfected GFP-tagged WT and K44A DynII in Panx1-overexpressing cells to investigate the role of dynamin in Panx1 internalization. Panx1 appeared to partially co-distribute with WT (Fig. 9A) and the K44A (Fig. 9C) DynII mutant at the plasma membrane. Upon BFA treatment, cells co-expressing Panx1 and WT DynII revealed consistent co-distribution at the cell surface with a slight increase in the intracellular localization of Panx1 (Fig. 9B). BFA-treated cells co-expressing Panx1 and the K44A DynII mutant revealed less intracellular Panx1 when compared with cells expressing only Panx1 in the same culture environment (Fig. 9D). To determine whether dynamin may in fact regulate Panx1 internalization, we assessed the effect of the WT and K44A DynII mutant overexpression on the glycosylation species of Panx1. It was predicted that if dynamin GTPase plays a role in Panx1 internalization, then an increase in the Gly2 expression of Panx1 would be seen in the presence of the K44A DynII mutant or dynasore. In the untreated group, expression of Panx1 alone or in presence of the WT or K44A DynII revealed no significant difference among all the three glycosylated species of Panx1 (Fig. 9, E and F). In contrast, BFA treatment significantly reduced the Gly2 species with a subsequent increase in the Gly1 species of Panx1 in all groups (Fig. 9, E and F). In untreated cells, when compared with the WT DynII, no significant increase in the Gly2 species of Panx1 was observed with K44A DynII co-expression (Fig. 9, E and F). As controls, the overexpression of the GFP-tagged WT and K44A DynII revealed a band at ~125–130 kDa (Fig. 9E). When maintained at 4 °C, live cells expressing WT or K44A DynII revealed transferrin distribution at cell-cell appositions (Fig. 9G). Following incubation at 37 °C, the WT DynII showed a clear accumulation of transferrin in the intracellular compartment, whereas K44A DynII restricted the ability of transferrin uptake (Fig. 9G). Given the role of dynamin in vesicular budding (27), it was not surprising to find that Panx1 did not interact with DynII in co-immunoprecipitation studies (Fig. 9H).

The role of dynamin-mediated internalization was also assessed by treating Panx1-overexpressing cells with the pharmacological inhibitor of DynII, dynasore. Similar to DynII K44A, dynasore did not show any additive effect on the BFA-induced expression levels of the Panx1 Gly2 and Gly1 species (supplemental Fig. 3). Overall, our results indicate that Panx1 internalization is independent of dynamin.

**DISCUSSION**

The focus of this study was 2-fold as follow: first, to identify the role of the Panx1 C-terminal domain in regulating Panx1 oligomerization and trafficking, and second, to elucidate the mechanisms and pathways responsible for the internalization and degradation of Panx1. First, we discovered that the Panx1 mutant lacking the C-terminal was incapable of trafficking to the cell surface. Second, during the retention of the mutant in the ER, it was processed to the high mannose species but had reduced ability to interact with full-length Panx1, suggesting that the C-terminal domain partially regulates Panx1 oligomerization. Third, quality control mechanisms targeted mutant Panx1 to premature degradation via proteasomes. Fourth, Panx1 channels at the cell surface appear to be long lived and upon internalization are destined for lysosomal degradation. Finally, the internalization of Panx1 was found to be independent of endocytic machinery that involved clathrin, caveolins, and dynamin GT Pases. Thus, we conclude that the physiologi-
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cal role for Panx1 channels may be governed more readily by events that regulate channel gating rather the events that govern channel assembly and degradation as has been well documented for the connexin family of gap junction proteins.

Regulatory Roles of the C-terminal Domain—Panx1 undergoes oligomerization into a hexamer and traffics to the plasma membrane where it functions as a channel (14, 28, 29). However, the domains of Panx1 that regulate its oligomerization have not been resolved. Quality control mechanisms dictate that proper folding and oligomerization is necessary for molecules to escape the ER and proceed along the secretory pathway (30). In the case of mutant Panx1, we found that, although it could readily be glycosylated to the high mannose containing species, its ability to oligomerize or interact with full-length Panx1 was greatly compromised and thus it was subject to endoplasmic reticulum-associated degradation via proteasomes. It is well established that misfolded or improperly assembled proteins retained in the ER are destined for premature degradation if molecular chaperones are unable to correct their folding (31, 32). Not surprisingly, mutant Panx1 further failed to be trafficked along the secretory pathway to its functional destination at the plasma membrane. ER-based chaperones, such as calnexin, are well known to mediate quality control by aiding in the proper folding of glycoproteins, while restricting the trafficking of misfolded proteins (33). Clearly, calnexin co-localization with the mutant Panx1 in our study further supports our proposal that the key mechanism regulating its retention within the ER is likely rooted in its failure to properly fold and oligomerize. Misfolded mutant proteins often impair cell surface trafficking of their wild type counterparts. This was also the case in this study as the mutant Panx1 clearly reduced the levels of wild type Panx1 found at the cell surface.

Interestingly and consistent with a study by Boassa et al. (14), our studies suggest that full-length Panx1 is destined for lysosomal degradation. In keeping with our current findings, we propose a model where full-length Panx1 is preferentially destined to lysosomes, as opposed to proteasome-dependent degradation of mutant Panx1.

Fate of Cell Surface Panx1—Internalization of K\(^{+}\) (34), Ca\(^{2+}\) (35), Cl\(^{-}\) (36), and gap junction channels (23, 37, 38) is mediated by clathrin and/or caveolin-driven pathways; however, this does not appear to be the case for Panx1 channels. Our data indicate that although Panx1 can co-exist and co-distribute in the same cell surface microdomain as clathrin, AP2, caveolins, and dynamin, its physical interaction with protein networks that contain any of these members of the endocytic machinery could not be detected. Likewise, dissolving lipid membrane rafts failed to alter the status of Panx1 at the cell surface. Moreover, dominant-negative and drug inhibition of dynamin GTPase also failed to alter the cell surface status of Panx1.

Previous studies have shown that when de novo Panx1 delivery to the cell surface is blocked with an inhibitor of the secretory pathway, brefeldin A, the clearing of preexisting Panx1 from the cell surface is slow (15, 17). Thus, these studies would infer that the half-life of Panx1 channels at the cell surface is long and in keeping with previously studied integral membrane protein, such as P2X7R, which functions at the cell surface and exhibits a half-life of 54 h (39). Taken together, Panx1 channel internalization is a less common occurrence than what is observed for more dynamic channels such as connexin gap junction channels (40). Consequently, the lack of evidence that Panx1 internalizes via caveolin-, clathrin-, or dynamin-dependent pathways may be rooted in the fact that these channels are either very long lived or they are subject to a nonclassical internalization pathway that has yet to be defined.

Pathophysiological Significance of Elucidating the Functional Domains of Panx1 and Its Life Cycle—It is becoming increasing well understood that Panx1 plays many important roles in cell and tissue function. Intriguingly, Panx1 knock-out mice have no obvious anatomical or tissue abnormalities (12), which has led to the suggestion that Panx1 channels are either not critical for normal tissue and cell health or other pannexin family members or related channels such as connexin hemichannels are able to compensate for the loss of Panx1. With growing reports of Panx1 forming large pore channels implicated in neuronal necrosis (9), epileptic seizure-like activity (10), and initiation of the death complex with the P2X7 receptor (11), we suggest that Panx1 will have a central and critical role in several pathologies. Thus, a clear molecular understanding of the life cycle of Panx1 remains seminal as we begin to assess the role of Panx1 in disease.

In summary, our present findings highlight a clear functional role for the C-terminal domain of Panx1. We have previously shown that this domain directly binds actin (21) thus tying Panx1 into a number of actin-based molecular networks. We further show that the C terminus is required for proper Panx1 oligomerization and ultimate trafficking to the cell surface. At the cell surface, our study and other studies would collectively suggest that Panx1 channels are long lived (15, 17). Thus, it is likely that many physiological changes that regulate the function of Panx1 channels cause molecular events that are related to the opening and closing of channels and not to the formation and removal of channels as is often the case for connexin gap junction channels. Finally, when Panx1 channels sufficiently age, their internalization is governed by unique machinery that remains to be determined.

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