Alternative Splicing of the First Intracellular Loop of Plasma Membrane Ca\(^{2+}\)-ATPase Isoform 2 Alters Its Membrane Targeting*

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Plasma membrane Ca\(^{2+}\)-ATPases (PMCA) are involved in local Ca\(^{2+}\) signaling and in the spatial control of Ca\(^{2+}\) extrusion, but how different PMCA isoforms are targeted to specific membrane domains is unknown. In polarized MDCK epithelial cells, a green fluorescent protein-tagged PMCA4b construct was targeted to the basolateral membrane, whereas a green fluorescent protein-tagged PMCA2b construct was localized to both the apical and basolateral domain. The PDZ protein-binding COOH-terminal tail of PMCA2b was not responsible for its apical membrane localization, as a chimeric pump made of an NH\(_{2}\)-terminal portion from PMCA4 and a COOH-terminal tail from PMCA2b was targeted to the basolateral domain. Deletion of the last six residues of the COOH-terminus of either PMCA2b or PMCA4b did not alter their membrane targeting, suggesting that PDZ protein interactions are not essential for proper membrane localization of the pumps. Instead, we found that alternative splicing affecting the first cytosolic loop determined apical membrane targeting of PMCA2. Only the “w” form, which contains a 45-amino acid residue insertion, showed prominent apical membrane localization. By contrast, the x and z splice variants containing insertions of 14 and 0 residues, respectively, localized to the basolateral membrane. The w splice insert was the crucial determinant of apical PMCA2 localization, and this was independent of the splice configuration at the COOH-terminal end of the pump; both PMCA2w/b and PMCA2w/a showed prominent apical targeting, whereas PMCA2x/b, PMCA2z/b, and PMCA2z/a were confined to the basolateral membrane. These data report the first differential effect of alternative splicing within the first cytosolic loop of PMCA2 and help explain the selective enrichment of specific PMCA isoforms in specialized membrane compartments such as stereocilia of auditory hair cells.

Plasma membrane Ca\(^{2+}\)-ATPases (PMCA) constitute the major high affinity Ca\(^{2+}\) extrusion system of eukaryotic cells.

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§ The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\)-ATPases; MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole; NHERF2, Na\(^+/\)H\(^+\) exchanger regulatory factor-2; DPBS, Dulbecco’s phosphate-buffered saline; CM, Ca\(^{2+}\)/Mg\(^{2+}\).
epithelial cells where it may interact with the PDZ protein synapse-associated protein 97. By contrast, we found that a GFP-tagged PMCA2b isoform was mainly targeted to the apical membrane where it co-localized with NHERF2 as a specific interaction partner (9).

Here we first set out to test the hypothesis that alternative splicing affecting the COOH-terminal tail may lead to differential membrane targeting of PMCA2s. By using recombinant protein expression and confocal fluorescence microscopy, we find that the PDZ binding tail is dispensable for basolateral or apical membrane targeting, suggesting that COOH-terminal splicing plays no primary role for the proper localization of PMCA isoforms. Surprisingly, however, alternative splicing at site A influences apical or basolateral localization of PMCA2. PMCA2w, but not 2x or 2z, is targeted to the apical membrane, and this is true regardless of whether the COOH-terminal splice corresponds to the a or b variant. Our data suggest for the first time a role for alternative splicing at site A of PMCA2, indicating the importance of this splicing event for differential membrane targeting of the calcium pump in polarized cells.

**EXPERIMENTAL PROCEDURES**

**Construction of PMCA Expression Vectors—** GFP-PMCA4b encoding human PMCA4b fused at its NH2 terminus to GFP was generated by cloning an Xhol fragment carrying the full-length PMCA4b sequence into a modified pEGFP-C1 expression vector (Clontech). The PMCA4b sequence was PCR-amplified using primers 4b-forward and 4b-stop (5'-gag gcg gcc ggg ggt ggg aga aac acc caa gcc gct gtc cc-3') and 4b-stop (5'-ccc cct gga aaa etg tct tca gtt gtc cc-3') containing Xhol recognition sequences (underlined). The full-length coding sequence of PMCA2w/b was first assembled as a NotI fragment in pBluecript-KS using reverse transcriptase-PCR and cDNAs cloned previously (4). To generate full-length cDNAs for PMCA2w/b and PMCA2x/b, a BarEI-SphI fragment encompassing the alternative splice site A region in PMCA2x/b was replaced by corresponding cassettes containing the 2w or 2z sequence, respectively. The NotI fragments carrying the full-length PMCA2w/b, -2xb, or -2zb sequence were then excised from pBluecript-KS and subcloned into the NotI site of pSPORT-1 (Invitrogen). Finally, the PMCA2x/b sequences were cloned as MinI-SalI (2w/b and 2z/b) or MinI-KpnI fragments (2x/b) into the modified pMM4 expression vector (3) to generate pMMx-PMCA2w/b, pMMx-PMCA2x/b, and pMMx-PMCA2z/b. To create constructs for GFP-PMCA2w/b and GFP-PMCA2x/b, the full-length PMCA2w/b and PMCA2x/b sequences were released as SacII/XbaI fragments from the original pBluecript-KS vectors and cloned into a modified pEGFP-C1 expression vector (Clontech) containing consecutive hemagglutinin and His6 tags (10) between the GFP and the respective PMCA sequences. To make constructs GFP-PMCA2w/b and PMCA4b plasmid described previously (9) was used as starting material because it allowed access to a convenient downstream restriction site. First, an ~250-bp EcoRI-SalI fragment encoding the COOH-terminal 72 residues was replaced by a corresponding PCR-generated EcoRI-SalI fragment carrying a translational stop codon six residues upstream of the original terminus. An ~2.3-kb BglII fragment containing the NH2-terminal ~750 codons was then excised from this plasmid and replaced by corresponding BglII fragments from GFP-PMCA2w/b and GFP-PMCA2x/b to create the constructs GFP-PMCA2w/b and GFP-PMCA2x/b, respectively. The expression vector pMMx-PMCA2w/a has been described previously (11). The expression vector pMMx-PMCA2w/a was generated by a three-way ligation combining SacII-KpnI-digested pMMx plasmid DNA, an ~1.3-kb SalI-ScaI fragment from GFP-PMCA2w/b containing the 5' ending coding sequence including the "w" insert at splice site A, and an ~2.4-kb SacI-KpnI fragment from pMMx-PMCA2w/a (containing the 3' region and a-tail sequence at splice site C). The chimeric PMCA construct GFP-PMCA4x2btail was made by replacing an ~350-bp BamHI fragment specifying the COOH-terminal residues from 1100 to 1205 in GFP-PMCA4b with a BamHI fragment specifying the corresponding COOH-terminal sequence from plasmid GFP-PMCA2x/b (9). The final construct encodes a GFP-tagged chimeric PMCA consisting of residues 1–1000 of PMCA4 (x splice variant) fused to the COOH-terminal 112 residues of PMCA2 (b splice variant). The integrity of all constructs was confirmed by DNA sequencing in the Mayo Molecular Biology Core Facility. A schematic representation of all constructs is shown in Fig. 2.

**Expression of Recombinant PMCA2s and Immunoblotting—** COS-1 cells were grown to ~80% confluence on 6-well plates (Costar) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, non-essential amino acids, 1 mM sodium pyruvate, glutamine, and antibiotic/antimycotic mixture (all cell culture reagents from Invitrogen). Cells were transfected with 2 μg of plasmid DNA using LipofectAMINE Plus™ according to the manufacturer's instructions (Invitrogen). After ~48 h, the cells were rinsed, lysed as described (7), and the protein concentration in the lysates determined by the BCA assay (Pierce). About 30 μg of the lysates from untransfected (control) and transfected cells were separated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose following standard Western blotting...
procedures (12). Nitrocellulose membranes were blocked in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20 and 5% milk before detection of the PMCAAs using specific antibodies. Monoclonal Pan-PMCA antibody 5F10, monoclonal anti-PMCA4 antibody J9, and affinity-purified polyclonal anti-PMCA2 antibody NR-2 were obtained from John Penniston and Adelaida Filoteo (Mayo Clinic) and used at dilutions of 1:2000, 1:600, and 1:5000, respectively. Secondary goat anti-mouse or goat anti-rabbit antibodies coupled to horseradish peroxidase were purchased from Sigma and used at 1:5000 dilution. Incubation with primary and secondary antibodies, washing, and detection of the signals was done as described previously (13).

Confocal Fluorescence Microscopy—Type I MDCK epithelial cells (ATCC number CCL-34, Manassas, VA) were grown to confluence on glass coverslips in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected with a total of 2 µg of plasmid DNA using LipofectAMINE 2000™ (Invitrogen). 48 h after transfection, the cells were fixed for 15 min at room temperature in 4% paraformaldehyde (Tousimis, Rockville, MD) diluted in DPBS (Ca²⁺/Mg²⁺) (DPBS + Ca²⁺/Mg²⁺, Invitrogen). After five brief washes in DPBS + CM, coverslips were further fixed and permeabilized in pre-chilled methanol for 15 min at −20 °C. The cells were blocked in DPBS + CM containing 5% normal goat serum and 1% bovine serum albumin (blocking buffer) and were then incubated for 1 h at room temperature with affinity-purified polyclonal anti-PMCA2 antibody NR-2 (1:1000, final concentration 0.8 µg/ml) or monoclonal Pan-anti-PMCA antibody 5F10 (1:800). After washing 3 times for 5 min in DPBS + CM, the cells were incubated for 1 h at room temperature with the appropriate anti-rabbit or anti-mouse secondary antibodies coupled to Alexa 488 or Alexa 594 (Molecular Probes, Eugene, OR). The secondary antibodies were used at a dilution of 1:800, and all antibodies for immunofluorescence were diluted in blocking buffer. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Eugene, OR) was also added to the secondary antibody application at a dilution of 1:500 (final concentration 20 µg/ml) to stain nuclei. After final washing, coverslips were mounted in Prolong mounting media (Molecular Probes). Confocal micrographs were taken on a Zeiss LSM510 microscope using an Apochromat 63×/1.4 oil immersion objective and captured using LSM510 software version 2.8 (Zeiss). Images were imported and edited using Adobe Photoshop 5.0.

Quantification of Apical Membrane Fluorescence—MDCK cells were grown to confluence as a monolayer on glass coverslips, transfected with PMCA2 expression constructs and prepared for immunofluorescence as described above using anti-PMCA2 antibody NR-2 and Pan-PMCA antibody 5F10 followed by Alexa 488 and Alexa 594 secondary antibodies, respectively. Confocal images were captured on a Zeiss LSM510 microscope as mentioned above. Images of the apical most 2-µm section of 20 representative cells from each transfection were collected, applying the same detector gain, zoom, amplitude offset and gain, transmission percent, pinhole size, and scan speed for each image collected within the frame mode and using an average from two images. By using the circular-shaped fixed area tool, an arbitrary area was selected that encompassed a majority of the collected apical images of each cell without incorporating any of the area outside of the apical domain (such as the lateral “rim” of the cell). This fixed circle-shaped area was then placed on the apical domain of the apical images in order to collect the mean fluorescence intensity (using the green channel) per given surface area for each of the 20 images from each of the different transfections. Choosing a circle that encompasses most of the membrane is essential as it yields an unbiased sampling of the entire apical domain as a whole and negates any effects (such as sampling microdomains) that would skew the overall mean intensity for a given image. Each cell could then be represented by a number equal to the mean apical fluorescence intensity of that cell. These numbers were used to construct a line graph showing the mean intensity for each of the 20 cells from a given transfection and were also displayed in a bar graph showing the averages of the 20 mean fluorescence intensities for each of the PMCA2 constructs.

RESULTS

Differential Targeting of PMCA Isoforms in Polarized MDCK Cells—We noted previously that endogenous PMCA4b was almost exclusively expressed in the basolateral membrane of polarized MDCK cells (7, 14), whereas a significant amount of GFP-tagged PMCA2b was found at the apical membrane where it co-localized with its binding partner NHERF2 (9). To confirm that the observed difference in membrane targeting is not due to the NH₂-terminally added GFP moiety, we expressed GFP-PMCA4b in MDCK cells and compared its localization to that of GFP-PMCA2b. Both GFP-tagged proteins were readily expressed in transiently transfected COS cells as determined by Western analysis using isoform-specific antibodies (Fig. 3).

When expressed in polarized Madin-Darby canine kidney cells, GFP-PMCA4b was still targeted to the basolateral membrane (Fig. 4A), whereas the GFP-PMCA2b construct was prominently present at the apical membrane in addition to labeling the basolateral membrane (Fig. 4B). Thus, the difference in membrane localization of these two PMCA isoforms is not due to the presence of the GFP moiety at their NH₂ terminus.

Differential Membrane Targeting of PMCA2b and PMCA4b Is Not Due to Their COOH-terminal Tails—Different PMCA b splice variants interact promiscuously with several PDZ proteins but also recognize specific PDZ protein partners (7, 9). For example, PMCA2b, but not PMCA4b, binds to NHERF2 which is a member of a small subfamily of PDZ proteins involved in membrane trafficking, anchoring, and regulation of ion transporters and receptors including Na⁺/H⁺ exchanger-3 (15), β2-adrenergic receptor (16), and the cystic fibrosis transmembrane conductance regulator (17, 18). The differential localization of PMCA2b and PMCA4b might therefore be dependent upon different protein-protein interactions by their COOH-terminal tails. To test this hypothesis, we generated a chimeric construct (GFP-PMCA4x2btail, see Fig. 2) in which the GFP-PMCA4b COOH-terminal tail downstream of residue 1100 was replaced by the corresponding tail of PMCA2b. When expressed in polarized MDCK cells, the localization of this chimera was indistinguishable from GFP-PMCA4b, i.e. the chimeric pump was exclusively found in the basolateral membrane (Fig. 5). Thus, the presence of the PMCA2b COOH-tail does not impart apical targeting on a chimeric protein with its NH₂-terminal segment derived from PMCA4.

Alternative Splicing at Site A Determines Differential Membrane Localization of PMCA2b Variants—Although PMCA2 and PMCA4 differ at many sequence positions throughout their length (their overall identity amounts to about 75%), the most

FIG. 3. Expression of recombinant PMCA constructs. Western blots are shown of protein lysates from COS cells transfected with various GFP-tagged (top panel) or untagged (bottom panel) PMCA constructs as indicated on top of each lane and as illustrated in Fig. 2. Equal amounts of cell lysates (~30 µg) were separated on 7.5% denaturing polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. The membranes were probed with antibodies against PMCA2 to detect GFP-tagged PMCA2w/b, -2x/b, and -2z/b as well as untagged PMCA2w/b and -2a/b, with antibodies against PMCA4 to detect GFP-tagged PMCA4x/b, or with a Pan-PMCA antibody to detect the GFP-PMCA4x2btail chimera, the non-tagged PMCA2w/b, -2x/b, and -2a/b as well as the endogenous COS-cell PMCA2 (untransfected control). The position of an ~130-KDa marker protein is indicated on the left. All constructs expressed a recombinant protein of the expected size, and all constructs were expressed at comparable levels.
MDCK cells were transfected with the GFP-PMCA4x/b-tail construct. GFP-PMCA2w/b shows prominent apical localization (apical section height) with virtually no staining of the apical membrane (baso-) lateral membrane. This is obvious from the x:z section shown below the en face views. Basolateral localization of a PMCA4-PMCA2b chimera. MDCK cells were transfected with the GFP-PMCA4x/b-tail construct encoding a GFP-tagged chimeric PMCA consisting of a PMCA4 NH2-terminal portion fused to the COOH-terminal portion from PMCA2b. After fixing, the cells were incubated with Pan-PMCA antibody 5F10 and an Alexa 594-labeled secondary antibody to visualize all PMCA constructs without any effect of the NH2-terminal GFP moiety, these vectors were made without any tag to represent the wild-type full-length PMCA2 variants. Western blots of equal amounts of total cell lysate from transfected COS cells showed that all constructs were expressed at comparable levels (Fig. 3). When expressed in polarized MDCK cells, PMCA2x/b and PMCA2z/b were primarily localized in the basolateral membrane (Fig. 6, middle and right panels), whereas PMCA2w/b showed prominent apical localization in addition to basolateral staining (Fig. 6, left panel). This difference in membrane localization was readily seen by taking confocal "en face" sections at different depths of the cells and is best illustrated in the x:y images shown in Fig. 6. Because all three constructs correspond to the b splice variant at their COOH-terminal end and are identical except for the insertion of 45 (w-form), 14 (x-form), or 0 (z-form) amino acid residues in their first intracellular loop, the observed differences in membrane localization must be due to the changes that occurred at splice site A, specifically to the insertion of 31 extra residues in the w splice form.

The PDZ Protein-binding COOH-terminal Sequence Is Not Required for Differential Membrane Targeting of PMCA2 Splice Site A Variants. The experiment using a chimeric GFP-PMCA4x/b-tail construct (see Fig. 5) had already shown that the COOH-terminal tail is not the major determinant for apical versus basolateral targeting of the PMCA. However, the presence of a functional PDZ-binding sequence may still be required for faithful membrane targeting of the pumps. To test if this is the case, we expressed GFP-PMCA2w/b and GFP-PMCA2x/b constructs lacking their six COOH-terminal residues (Figs. 2 and 3), thus rendering them unable to interact with PDZ proteins. As shown in Fig. 7, GFP-PMCA2w/bΔ6 and GFP-PMCA2x/bΔ6 were still faithfully targeted to the apical and basolateral membranes, respectively, as predicted from their splice site A configuration. Similarly, GFP-PMCA4x/bΔ6 was targeted only to the basolateral membrane (not shown), as had been observed for the corresponding full-length PMCA4x/b isoform (Fig. 4A). We conclude that a functional PDZ-binding domain at the COOH terminus is not required for targeting of the PMCA and hence that PDZ protein interactions are dispensable for directing the pumps to their membrane destination.

The w Insertion at Splice Site A Determines Apical Membrane Targeting of PMCA2 Isoforms. If the insertion of the 31
The splice site A configuration determines the membrane localization of PMCA2 irrespective of the splice site C configuration. Full-length PMCA2w/a and PMCA2z/a were expressed in MDCK cells, and their localization was studied by confocal fluorescence microscopy. Detection of recombinant PMCAs was as described in the legend to Fig. 6. DAPI-stained nuclei appear blue. Note that the w splice form of PMCA2a shows prominent localization in the apical membrane (left panel), whereas the z splice form is confined to the basolateral membrane (right panel) as was found for the localization of PMCA2w/b and PMCA2z/b, respectively (see Fig. 6). Corresponding xz sections are shown below each en face view. Arrows indicate the height at which the en face images were captured.

**DISCUSSION**

Dynamic and spatially segregated changes in Ca\(^{2+}\) form the basis for the exquisite specificity of intracellular Ca\(^{2+}\) signaling. This is achieved through subcellular compartmentalization and expression of an appropriate “tool kit” of Ca\(^{2+}\) transporting, buffering, and signaling molecules (19, 20). Although the PMCA isoforms and splice variants are differentially expressed and localized in several tissues and cell types. For example, auditory and vestibular hair cells of the inner ear almost exclusively express PMCA2a in their apical stereocilia. In the basolateral membrane, these same cells express mainly PMCA1b (25). Similarly, in the retina of the mouse, PMCA1 is highly abundant in the synaptic terminal and inner segment of photoreceptors but absent from the outer segment, and PMCA2 is concentrated in the synaptic membrane of rod bipolar cells in the inner plexiform layer (26).
Differential localization in distinct membrane domains may be one of the distinguishing features among PMCA isoforms and splice variants. Because the most pronounced difference between PMCA splice variants occurs between the a and b COOH-terminal splice variants, it was tempting to speculate that their different tails carry the information for differential membrane targeting. Moreover, only the b variants are known to interact with PDZ domains (7, 27), and PDZ protein interactions have been shown to be involved in the targeting, retention, and/or anchoring of several membrane receptors and ion channels (28, 29). Contrary to findings with the cystic fibrosis transmembrane conductance regulator and other transporters (30–32), our results show that the targeting of PMCAs in polarized MDCK cells is independent of their splice site C configuration and does not require a functional PDZ-interacting COOH-terminal tail. Instead, we have uncovered an important role for changes at splice site A in directing PMCA2 variants to the apical versus basolateral membrane. Only the variant with the largest insert of 45 residues (PMCA2w) was apically directed, whereas PMCA2x (14-residue insert) and PMCA2z (no extra amino acids inserted) were localized in the basolaterally directed, whereas PMCA2x (14-residue insert) and PMCA2z (no extra amino acids inserted) were localized in the apical membrane of epithelial cells where it is believed to play an essential role in transcellular Ca\(^{2+}\) flux. Thus, alternative RNA splicing affecting the first cytosolic loop of the pump (37) (see Fig. 1) may be involved in apical targeting or apical protein retention. A differential yeast two-hybrid screen with the first cytosolic loop of the w and x (or z) splice variants as bait may provide an attractive future approach to this problem.

Finally, alternative splicing at site A of PMCA2 appears to be highly regulated. In IMR32 neuroblastoma cells, a specific and rapid switch from the 2w to the 2x variant occurs upon KCl depolarization. This splice switch is dependent on a rise in intracellular Ca\(^{2+}\), is independent of new protein synthesis, and can be observed with a 30–45 min of cell stimulation (38). Although differential membrane distribution of PMCA2x and 2w variants has not been investigated in IMR32 cells, it is of interest that depolarization and a rise in Ca\(^{2+}\) are triggers of (neuronal) differentiation. Perhaps the alternative splice switch at site A of PMCA2 allows the cell to quickly re-distribute PMCA2 to membrane domains where they are needed. In neurons, this could be at newly forming synaptic sites in the dendrites. In epithelial cells of the mammary gland or the distal kidney, the PMCA2w splice form may be targeted to apical membranes in response to hormonal regulation of transcellular Ca\(^{2+}\) flux. Thus, alternative RNA splicing affecting the first cytosolic loop of PMCA2 appears to be a means for the regulated re-deployment of this Ca\(^{2+}\) pump to different membrane domains.

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