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A C-terminal di-leucine motif controls plasma membrane expression of PMCA4b

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ABSTRACT

Recent evidences show that the localization of different plasma membrane Ca^{2+} ATPases (PMCAs) is regulated in various complex, cell type-specific ways. Here we show that in low-density epithelial and endothelial cells PMCA4b localized mostly in intracellular compartments and its plasma membrane localization was enhanced upon increasing density of cells. In good correlation with the enhanced plasma membrane localization a significantly more efficient Ca^{2+} clearance was observed in confluent versus non-confluent HeLa cell cultures expressing mCherry-PMCA4b. We analyzed the subcellular localization and function of various C-terminally truncated PMCA4b variants and found that a truncated mutant PMCA4b-ct24 was mostly intracellular while another mutant, PMCA4b-ct48, localized more to the plasma membrane, indicating that a protein sequence corresponding to amino acid residues 1158–1181 contained a signal responsible for the intracellular retention of PMCA4b in non-confluent cultures. Alteration of three leucines to alanines at positions 1167–1169 resulted in enhanced cell surface expression and an appropriate Ca^{2+} transport activity of both wild type and truncated pumps, suggesting that the di-leucine-like motif ^{1167}LLL was crucial in targeting PMCA4b. Furthermore, upon loss of cell–cell contact by extracellular Ca^{2+} removal, the wild-type pump was translocated to the early endosomal compartment. Targeting PMCA4b to early endosomes was diminished by the $L^{1167-69}A$ mutation, and the mutant pump accumulated in long tubular cytosolic structures. In summary, we report a di-leucine-like internalization signal at the C-tail of PMCA4b and suggest an internalization-mediated loss of function of the pump upon low degree of cell–cell contact.

1. Introduction

The Ca^{2+} signal is finely tuned by concerted actions of channels (that switch on the Ca^{2+} signal) and pumps (that switch it off) located in intracellular compartments as well as in the plasma membrane [1]. Both switch mechanisms are tightly controlled in space and time to meet specific needs of the cells. One of the main "off" mechanisms of the Ca^{2+} signal is the plasma membrane Ca^{2+} ATPase (PMCA) present in all kinds of cells [2,3]. Four separate genes code for PMCA isoforms 1–4 and alternative splicing of the primary transcripts further increases the number of PMCA variants to over 20 [4,5]. The PMCA isoforms differ significantly in their regulatory and kinetic properties [3,6]. Therefore, they have the ability to control different cellular functions [7]. Activity of PMCAs is achieved at 3 levels (1) PMCAs can be regulated at the transcriptional level [8]. Moreover, influencing the transcription of certain types of PMCAs can generate complex changes in the expression of other PMCAs and Ca^{2+} signaling systems [9]. (2) The second level of PMCA activity regulation is post-transcriptional modification of the protein by calmodulin-binding, phosphorylation and/or proteolysis [10]. (3) The third level of activity control is changing the subcellular localization of PMCA. Adjusting the density of PMCA in particular plasma membrane compartments substantially contributes to the specific outcome of the Ca^{2+} signal. Recent experiments from our laboratory and others suggested that the "w" splice is essentially important in the apical localization of the PMCA2w isoform in polarized MDCK cells [11,12] or in the stereocilia of hair cells [13]. Our experiments also showed that co-expression with the Na^+/H^+ exchanger regulatory factor 2 (NHERF2) greatly enhanced the apical localization of PMCA2w/b [14] the prominent pump of the lactating mammary gland [15]. NHERF2 was also required for the agonist-induced plasma membrane translocation of PMCA in HT-29 human colonic epithelial cells [16].

PMCA4b has the lowest basal activity and the slowest activation rate of all PMCAs [6]. This is the pump of cells derived from hematopoietic stem cells and in cells of epithelial origin; thus it is expressed mostly in non-excitable cells/tissues where it can handle relatively

Abbreviations: EEA1, early endosome antigen 1; ER, endoplasmic reticulum; MDCK, Madin Darby canine kidney; NHE, Na^+/H^+ exchanger; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; PDZ, PSD-95 Dlg ZO-1; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; WGA, wheat germ agglutinin

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slow Ca^{2+} changes. Recent publications have suggested that the expression of the PMCA4b isoform is increased during differentiation of cells [17,18]. These studies demonstrated upregulation of PMCA4 expression during confluence-mediated differentiation of colon-cancer cells. Changes in the localization pattern of the PMCA4 protein in post-confluent cultures in colon cancer cells [19] as well as in mouse distal convoluted tubule (mDCT) cells [20] have also been reported. Previously, we described enhanced plasma membrane localization of the PMCA4b pump in COS-7 and N2a cells elicited by a C-terminal PDZ interaction between the pump and the post synaptic density protein, PSD-95 [21]. Specific localization of PMCA4 was further established in cholesterol-rich lipid rafts in synaptosomes of pig cerebellum [22] and at the immunological synapse together with the ORAI channels and mitochondria [23].

An important way to influence cell signaling is the endocytic regulation of signaling proteins residing in the plasma membrane (receptors, channels and transporters) [24,25]. The proteins can be internalized either by clathrin-dependent or clathrin-independent pathways [26]. The internalization of proteins to endosomes is usually mediated by specific short sequence signal motifs including tyrosine-based sequences, dileucine-based motifs and single amino acid-specific sequences [27]. Di-leucine-based motifs are known to play role in the endocytosis and trafficking of various membrane receptors and proteins [28–33] (for a review, see [34]).

Little is known about the molecular mechanisms underlying the regulation of surface expression of the PMCAs, especially that of the PMCA4b isoform. Early localization studies using the fibroblast COS-7 cell line and C-terminal truncations/site-directed mutagenesis described an ER retention signal right upstream of the calmodulin-binding sequence between residues Glu^{1067} and Arg^{1087} of the PMCA4b pump [35]. This study described that removal of the C-terminal 118 residues resulted in exposing the retention signal so that the $PMCA4b_{\Delta 118}$ mutant retained in the ER compartment. Later we found that a C-terminally truncated PMCA4b-ct125 mutant localized correctly to the plasma membrane of fully polarized MDCK cells [36].

Here we show that increasing confluence of HeLa and HUVEC cells enhances plasma membrane localization of both the transiently and endogenously expressed PMCA4b pump. Detailed localization studies of C-terminally truncated mutant PMCA4b pumps uncovered a novel putative di-leucine-like motif downstream of the calmodulin-binding sequence. Further, we show that internalization of this pump upon low Ca^{2+} mediated loss of cell–cell contact occurs through the endosomal system that possibly uses the pathway of cadherins in HeLa cells.

2. Materials and methods

2.1. Chemicals and reagents

FuGene HD Transfection Reagent was obtained from Roche Applied Science. DMEM and OPTI-MEM were purchased from Gibco. LipofectAMINE was obtained from Invitrogen. Pierce Cell Surface Protein Isolation Kit (Prod. No. 89881) was obtained from Thermo Scientific. Pan-PMCA-specific mouse monoclonal antibody 5F10 [37] was used at a dilution of 1:5000 for immunoblotting and 1:250 for indirect immunofluorescence staining. PMCA4-specific mouse monoclonal antibody JA9 and PMCA4b-specific mouse monoclonal antibody JA3 were used at a dilution of 1:100 [37] . IgG1 isotype control antibody (Sigma-Aldrich) was used at dilution 1:100. Chicken polyclonal anti-Sodium Potassium ATPase (Abcam) and anti-pan-cadherin (Abcam) antibodies were used at a dilution of 1:250 for imunofluorescence staining. Anti-EEA1 (Abcam) was used at a dilution of 1:500. Wheat germ agglutinin (WGA) Alexa Fluor 633 conjugate (Molecular Probes), Alexa Fluor 488- and 594-conjugated goat anti-mouse IgG (Molecular Probes), Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa Fluor 647-conjugated goat anti-chicken IgG (Molecular Probes) antibodies were purchased from Invitrogen Corp. All other chemicals used were of reagent grade.

2.2. Construction of expression vectors

The truncated mutants (PMCA4b-ct24, -ct48, -ct57) have been previously generated by polymerase chain reaction and have been inserted into the expression plasmid pMM2 [37–39]. Suitable mutations were generated by site-directed mutagenesis using the overlap extension technique [40] utilizing the pMM2–PMCA4b [41] or the pMM2–PMCA4b-ct24 plasmids as a template. For each mutation, two separate DNA fragments having overlapping ends were amplified. Complementary internal primers used for the $L^{1167-69}A$ mutation were: forward 5′-CTAGGGTGGCAGCGGCGGATGGTG-3′ and reverse 5′-CACCATCCGCCGCTGCCACCCTAG-3′, internal primers used for the $L^{1147-48}A$ mutation were: forward 5′-CACCAGCCGCGGATGAGGAA-3′ and reverse 5′-TTCCTCATCCGCGGCTGGTG-3′, external primers for each mutation were: forward 5′-CTTCAATGAAATCAACTCCCG-3′ and reverse 5′-CGTCTCAGGTACACCGAGC-3′. The overlapping fragments containing the mutations were fused together in a subsequent extension reaction. The full-length constructs were ligated at the *Bbv*CI-*Hpa*I sites of the pMM2–PMCA4b or the pMM2–PMCA4b-ct24 plasmids. To create pEGFP–PMCA4b-$L^{1167-69}A$ construct, the mutation containing insert was cut out from the pMM2 plasmid with the *Pfl*MI and *Kpn*I restriction enzymes and the insert was ligated into the *Pfl*MI–*Kpn*I sites of the pEGFP–PMCA4b template plasmid [12]. To make the mCherry-PMCA4b construct, *Bam*HI and *Xho*I restriction sites were added to the ends of the mCherry sequence taken from a pcDNA3-mCherry plasmid (kind gift from Robert Katona, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences) by performing PCR with the following primers: forward 5′-CGAGCTCGGATCCACCATGGTGAGC-3′, reverse 5′-GCTCTCGAGTGCTTGTACAGCTCGTCC-3′. In this way the stop codon from the mCherry sequence was removed and a *Xho*I restriction site was added to it. This mCherry sequence was cloned into the pcDNA3.0 vector thus creating a new pcDNA3.0-mCherry vector containing a *Xho*I restriction site. The PMCA4b coding fragment was cut from the pEGFP–PMCA4b plasmid with *Xho*I and ligated into the *Xho*I site of the pcDNA3.0-mCherry vector. pN1-GCaMP2 plasmid was a kind gift of Junichi Nakai, RIKEN Brain Science Institute, Saitama, Japan [42].

2.3. Cell culture, transfection and Western blotting

HUVEC cells were kindly provided by the Institute of Pathophysiology, Semmelweis University, Budapest, Hungary. Cells were freshly isolated from human umbilical cord veins as described [43,44]. Cells from passages 2–4 were used for experiments. HeLa, MDCKII and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were transfected with the appropriate DNA constructs using the FuGENE HD (Roche) or LipofectAMINE (Invitrogen) transfection reagents according to the manufacturer's protocol. The PMCA expression was confirmed by Western blot analysis. Samples were resolved on 7.5% acrylamide gel following the procedure of Laemmli [45], with some modifications [21]. Proteins were subsequently transferred onto PVDF membranes and the blots were immunostained with the anti-PMCA antibody 5F10.

2.4. Membrane preparation from COS-7 cells

Crude microsomal membranes were prepared as described previously [46]. Briefly, cells were washed twice with ice-cold phosphate-buffered saline pH 7.4 and harvested in phosphate-buffered saline containing 0.1 mM AEBSF, 6 μg/ml aprotinin, 2.24 μg/ml leupeptin and 1 mM EGTA pH 7.4. Cells were collected by centrifugation and resuspended