The caspase-3 cleavage product of the plasma membrane Ca^{2+} -ATPase 4b is activated and appropriately targeted

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The calmodulin-activated transporter hPMCA4 (human plasma membrane Ca^{2+} -ATPase isoform 4) is a target for cleavage by caspase-3 during apoptosis. We have demonstrated that caspase-3 generates a 120 kDa fragment of this pump which lacks the complete autoinhibitory sequence [Paszty, Verma, Padanyi, Filoteo, Penniston and Enyedi (2002) *J. Biol. Chem.* **277**, 6822–6829]. In the present study we analysed further the characteristics of the fragment of hPMCA4b produced by caspase-3. We did this by overexpressing the caspase-3 cleavage product of hPMCA4b in COS-7 and MDCKII (Madin–Darby canine kidney II) cells. This technique made it possible to clearly define the properties of this fragment, and we showed that it is constitutively active, as it

forms a phosphoenzyme intermediate and has high Ca^{2+} transport activity in the absence of calmodulin. When this fragment of hPMCA4b was stably expressed in MDCKII cell clones, it was targeted without degradation to the basolateral plasma membrane. In summary, our studies emphasize that the caspase-3 cleavage product of hPMCA4b is constitutively active, and that the C-terminus is not required for proper targeting of hPMCA4b to the plasma membrane. Also, for the first time, we have generated cell clones that stably express a constitutively active PMCA.

Key words: activation, apoptosis, calmodulin, caspase-3, localization, plasma membrane Ca^{2+} -ATPase.

INTRODUCTION

PMCA (plasma membrane Ca^{2+} -ATPase or pump) is a ubiquitous Ca^{2+} transport protein that is essential for maintaining a low intracellular Ca^{2+} concentration. There are at least 20 different PMCA isoforms that are produced by four different genes and alternative splicing of the primary transcripts [1,2]. One place where these isoforms differ from one another is their C-terminus, which determines their specific responses to stimulation by calmodulin and their regulation by and interaction with other cellular signalling molecules [3,4]. This C-terminus has a high-affinity calmodulin-binding sequence which serves as an autoinhibitor of the Ca^{2+} -pumping activity. The extreme C-terminus of the b-type PMCA isoforms also interacts with PDZ domain-containing proteins [5–7].

Several lines of evidence suggest that removal of the C-terminus constitutively activates PMCA4b. Proteolytic digestion with a number of different proteases (trypsin, chymotrypsin, calpain, caspase-3) has demonstrated that fully active Ca-transporting fragments of PMCA4b could be generated by specific cleavage of its C-terminus [8–11]. Such cleavage by chymotrypsin, calpain or caspase-3 depended on the conformation of the pump, and was greatly enhanced when proteolysis occurred in the presence of calmodulin [12]. We also constructed a truncated form of hPMCA4b (human PMCA4b), hPMCA4b-ct120 (a construct that is truncated by 120 residues at the C-terminus), which ended just before the calmodulin-binding sequence, and demonstrated that it was constitutively active [13]. These observations led to the conclusion that the C-terminus serves as a built-in inhibitor which

folds back and binds to the main cytosolic domains, inhibiting catalytic activity in the absence of calmodulin.

More recently, we showed that hPMCA4b has a consensus sequence for cleavage by caspase-3, i.e. Asp¹⁰⁷⁷-Glu-Ile-Asp¹⁰⁸⁰ [10]. Caspase-3 cuts hPMCA4b immediately after Asp¹⁰⁸⁰, removing the 125-residue non-catalytic C-terminal tail and producing a 120 kDa (1080-residue) catalytic fragment. We demonstrated that this modification increased the basal activity, whereas it diminished stimulation by calmodulin. Another consequence of this cleavage is the removal of the PDZ-interacting motif from the C-terminus, which will affect specific molecular interactions involving the C-terminal region of PMCA4b and PDZ domain-containing proteins, such as members of the MAGUK (membrane-associated guanylate kinase) family [5,6], nitric oxide synthase I [14] and the calcium/calmodulin-dependent serine protein kinase (CASK) [15].

Recently, several research reports and review articles have suggested that the 120 kDa caspase-generated fragment of PMCA4b is inactive or has reduced activity [16–20]. All of these reports refer to a single turnover experiment [21] that showed reduced phosphoenzyme formation after treatment by caspase-3 of Sf9 cell membrane preparations expressing PMCA4b.

Another important aspect of PMCA function is its correct localization in the plasma membrane. An earlier report suggested that C-terminal truncation exposes a sequence in PMCA4 that targets the truncated pump to the endoplasmic reticulum, where it is degraded further [22]. Internalization of PMCA4b has also been reported after staurosporine treatment of CHO cell clones expressing PMCA4b [21].

Abbreviations used: DPBS, Dulbecco's modified PBS; EP, phosphorylated intermediate; MDCK, Madin–Darby canine kidney; PMCA, plasma membrane Ca^{2+} -ATPase; hPMCA, human PMCA; hPMCA4b-ct125, hPMCA4b construct truncated by 125 residues at the C-terminus.

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Ca^{2+} is an important player in apoptosis and many other signalling events, and PMCA is a key element of Ca^{2+} homeostasis. Therefore it is of great interest to demonstrate clearly whether PMCA4 is activated or inactivated following cleavage by caspase, and whether this cleavage promotes its internalization and further degradation. In order to characterize further the caspase-3 cleavage product of hPMCA4b, we made a plasmid that coded for a truncated PMCA4 that lacks all residues downstream of the caspase-3 cut site and thus corresponds to the apoptotic fragment. We overexpressed the wild-type and truncated hPMCA4b proteins in COS-7 and MDCKII (Madin–Darby canine kidney II) cells. Further, we selected MDCKII cell clones stably expressing the mutant protein. We analysed the phosphoenzyme formation and Ca^{2+} transport activity of the overexpressed proteins, and studied their localization in fully polarized MDCKII cell clones by laser scanning confocal microscopy. Our studies show that the caspase-3 cleavage product of hPMCA4b is stable, is properly targeted and is fully active even in the absence of calmodulin.

EXPERIMENTAL

Materials

Calmodulin was obtained from Sigma. Recombinant caspase-3 was from Upstate Biotechnology. Lipofectamine™ and OPTI-MEM were obtained from Life Technologies, Inc. Chicken polyclonal anti- Na^+/K^+ -ATPase antibody was from Chemicon International (Temecula, CA, U.S.A.), Alexa Fluor 488-conjugated goat anti-(mouse IgG) and Alexa Fluor 594-conjugated goat anti-(chicken IgG) were obtained from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals used were of reagent grade.

Construction of hPMCA4b-ct125 and expression vectors

PCR was used to create a construct that is truncated by 125 residues at the C-terminus. hPMCA4-ct125 terminates immediately after Asp¹⁰⁸⁰, the C-terminal residue of the caspase-cleaved hPMCA4. PCR was carried out with the primer pair 5'-TG-ACAACATCAACACAGCCC-3' and 5'-GGTACCTCAGTCAA-TCTCATCCAGTCCC-3'. The forward primer is situated upstream of a unique XmaI site. A termination codon (underlined) and a KpnI site were added as a 5' overhang to the reverse primer. The PCR product was cloned into pCR Blunt II TOPO vector (Invitrogen) and sequenced. The truncated insert was excised from pCR Blunt II TOPO with XmaI and KpnI. The excised insert was ligated to the vector fragment of XmaI/KpnI-digested full-length hPMCA4b in pMM2 (the pMM2 vector was originally called pMT2-m [23]). For the expression of hPMCA4 in MDCKII cells, a KpnI/SalI DNA fragment containing the complete hPMCA4 open reading frame was inserted as a blunt-ended fragment into the retroviral vector SPs-neo. SPs-neo was constructed from the original retroviral vector SPsLDS [24,25] by inserting a StuI/BstBI DNA fragment containing the neomycin resistance gene.

Expression of recombinant PMCA4s and immunoblotting

COS-7 and MDCKII cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. All cells were kept at 37°C and 5% CO_2 in a humidified atmosphere.

COS-7 cells were transfected with pMM2 plasmid DNA as described previously [10,12] using the Lipofectamine™ reagent, based on the protocol recommended by the manufacturer (Life Technologies, Inc.).

For retroviral transduction of MDCKII cells, Phoenix-Ampho retroviral packaging cells were transfected with the SPs-neo retroviral vector using the calcium phosphate co-precipitation kit (Life Technologies, Inc.). At 48 and 72 h after transfection, the virus-containing supernatant was collected and used immediately to transduce MDCKII cells. For transduction, 5×10^4 MDCKII cells were seeded into 24-well plates, and on the next day virus-containing supernatant, diluted 2-fold with fresh medium and containing 6 µg/ml Polybrene, was added. After centrifugation (1000 g, 90 min), cells were incubated at 37°C. Single-cell clones were isolated by limiting dilutions of transduced cells on 96-well plates.

Crude microsomes from cells were prepared as described previously [10,12]. The level of hPMCA4 expression was tested by Western blot analysis. The samples were electrophoresed on a 7.5% (w/v) acrylamide gel following the procedure of Laemmli [25a], except that the sample buffer contained 62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5 mM EDTA, 100 mM dithiothreitol and 125 mg/ml urea. The samples were subsequently electroblotted and the blots were immunostained by monoclonal antibodies 5F10 or JA9 [26].

In vitro digestion with recombinant caspase-3

Portions of 20 µg of microsomes isolated from COS-7 cells transfected with the appropriate construct were preincubated for 3 min at 37°C in 100 µl of medium containing 100 mM KCl, 25 mM Tes/triethanolamine, pH 7.2, 0.09 mM EGTA, 8.5% (w/v) sucrose, 5 mM dithiothreitol, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 0.1 mM CaCl_2 (10 µM free Ca^{2+}) and 235 nM calmodulin. Proteolysis was started by the addition of 0.5 µg of recombinant caspase-3 and stopped by the addition of ice-cold trichloroacetic acid (6% final concn). The precipitate was supplemented with 100 µg of BSA, washed once with distilled water, and then dissolved in electrophoresis sample buffer. Alternatively, the formation of phosphorylated intermediates and the Ca^{2+} transport activity of the digested membrane preparation were tested.

Activity measurements

Ca^{2+} transport was measured as described previously [10] in a reaction mixture containing 100 mM KCl, 25 mM Tes/triethanolamine, pH 7.2, 7 mM MgCl_2 , 100 µM CaCl_2 (labelled with ^{45}Ca), 40 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.2, 200 nM thapsigargin, 4 µg/ml oligomycin and 235 nM calmodulin. To obtain the desired Ca^{2+} concentration, various amount of EGTA were added to the reaction mixture. The free Ca^{2+} concentration was calculated with MaxChelator (<http://www.stanford.edu/~cpatton/maxc.html>). To ensure that digestion by caspase-3 was arrested during the transport assay, 5 µg (25 µl) of digested membranes was diluted into 200 µl of reaction medium. Ca^{2+} uptake was initiated by the addition of 5 mM ATP. The reaction was terminated by rapid filtration of the microsomes using Millipore membrane filters (0.45 µm pore size).

The phosphorylation reaction was carried out at 4°C in a medium containing 100 mM KCl, 25 mM Tes/triethanolamine, pH 7.2, 5 mM dithiothreitol, 100 µg CaCl_2 , 400 nM thapsigargin and 100 µM LaCl_3 . The reaction was initiated by the addition of 0.3 µM [γ - ^{32}P]ATP and terminated after 60 s by the addition of 6% trichloroacetic acid containing 7 mM P_i and ATP. The precipitate was supplemented with 100 µg of BSA, washed once with distilled water, and then dissolved in electrophoresis sample buffer. Gel electrophoresis of 4 µg of the phosphorylated samples was performed on acidic gels, as described in [11].

After electrophoresis, gels were dried and were subjected to autoradiography.

Immunocytochemistry

MDCKII cells were grown on eight-well Nunc Lab-Tek Chambered Coverglasses (Nalge Nunc International) coated previously with 0.03 mg/ml VitroGene (Cohesion Technology). For immunocytochemical detection, MDCKII cells were fixed for 5 min at room temperature in 4% (v/v) paraformaldehyde in DPBS (Dulbecco's modified PBS). After five brief washes with DPBS, samples were further fixed and permeabilized in pre-chilled methanol for 5 min at -20°C . MDCKII cells were then blocked for 1 h at room temperature in DPBS containing 2 mg/ml BSA, 1% fish gelatin, 0.1% Triton-X 100 and 5% goat serum. Samples were then incubated for 1 h at room temperature with mouse monoclonal anti-PMCA4b antibody (5F10; diluted 100-fold in blocking buffer) and a chicken polyclonal anti- $\text{Na}^{+}/\text{K}^{+}$ -ATPase antibody (diluted 500-fold in blocking buffer). After three washes in DPBS, cells were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-(mouse IgG) (H + L) and Alexa Fluor 594-conjugated goat anti-(chicken IgG) (H + L), both diluted 250-fold in blocking buffer. After repeated washes, samples were studied under an Olympus FV500-IX confocal laser scanning microscope using an Olympus PLAPO $\times 60$ (1.4) oil-immersion objective. For green and red fluorescence acquisitions, the samples were illuminated with 488 and 543 nm laser lines respectively, and confocal images were taken at 505–545 nm and > 560 nm respectively.

RESULTS AND DISCUSSION

Activity of the hPMCA4b fragment generated by *in vitro* digestion by caspase-3

First we analysed the characteristics of the caspase fragment generated from full-length hPMCA4b by *in vitro* digestion of microsomes isolated from hPMCA4b-overexpressing COS-7 cells using recombinant caspase-3. In our previous experiments we demonstrated that the cleavage of hPMCA4b is highly conformation-dependent [12]. Therefore, in the present study, digestion by caspase-3 was performed in the presence of calmodulin to convert the intact pump into the 120 kDa fragment (Figure 1). No degradation of the pump could be detected if it was incubated in the absence of the protease (Figure 1A); thus, the fragmentation shown in Figure 1(B) can fully be attributed to caspase-3.

In order to test the function of the caspase-generated fragment, we tested whether the fragment retains the ability to form a phosphorylated intermediate (EP). After digestion by caspase-3, samples were placed on ice, followed by the addition of lanthanum and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Lanthanum is known to enhance EP formation by blocking the dephosphorylation cycle of PMCA [27]. In contrast with the experiment described by Schwab et al. [21], a strong EP band was formed even after a 60 min incubation with caspase-3 (Figure 1C), which is clearly attributed to the 120 kDa fragment shown on the immunoblot (Figure 1B).

Then we tested ATP-dependent Ca^{2+} uptake by the caspase-3-treated microsomes. This assay has the advantage of measuring the entire catalytic cycle of the enzyme under conditions approaching the physiological ones. Figure 1(D) shows that proteolysis by caspase-3 did not inactivate the enzyme: when the Ca^{2+} transport activities were measured in the presence of calmodulin, the proteolysed and unproteolysed samples had essentially the same activity. These experiments show that the proteolytic fragment retained the full catalytic activity of PMCA4b.

Figure 1 *In vitro* digestion by caspase-3 of microsomes from COS-7 cells expressing hPMCA4b

Digestion was performed in the presence of calmodulin, and EP formation and Ca^{2+} transport activity were measured on the digested samples. Portions of 20 μg of microsomes were digested with recombinant caspase-3 in the presence of 235 nM calmodulin for the indicated times. After digestion, the samples were put on ice and EP formation was carried out by the addition of 100 μM LaCl_3 and 0.3 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 4°C for 1 min. (A) Immunoblot of 1 μg of microsomes incubated without caspase-3, stained by anti-PMCA antibody 5F10. (B) Immunoblot of 1 μg of the digested microsomes at each time point, stained by anti-PMCA antibody 5F10. (C) Autoradiography of 4 μg of the digested microsomes after phosphorylation. (D) Ca^{2+} transport activity of the caspase-digested and non-digested microsomes in the presence of calmodulin at the same time points as in (A) and (B). Values are means $\pm$ S.D. for three independent experiments.

Expression and activity of hPMCA4b-ct125 in COS-7 cells

To establish further that the caspase-3 cleavage product of hPMCA4b is fully and constitutively active, we constructed a C-terminally truncated mutant, hPMCA4b-ct125, that terminates immediately after the caspase-3 cleavage site Asp-Glu-Ile-Asp¹⁰⁸⁰. This mutant lacks all 125 residues downstream of the caspase-3 cleavage site (i.e. the entire calmodulin-binding regulatory region) and represents the caspase-3 cleavage product of hPMCA4b. Initially we overexpressed the wild-type and truncated forms of hPMCA4b in COS-7 cells. Microsomes from these cells were analysed by immunoblotting using antibodies 5F10 or JA9 [26]. 5F10 recognizes a linear epitope from residues 719–738 that is common to all known hPMCA gene products, while monoclonal antibody JA9 reacts specifically with PMCA isoform 4 near the

Figure 2 Expression of a truncated mutant corresponding to the caspase-3 cleavage product of hPMCA4b

(A) Left panel: immunoblot of microsomes from COS-7 cells transfected with empty vector (lane 1, 0.5 μg of microsomes; lane 2, 10 μg), hPMCA4b (0.5 μg ; lane 3) or hPMCA4b-ct125 (0.5 μg ; lane 4), stained by anti-PMCA antibody 5F10. Right panel: EP formation by microsomes from COS-7 cells transfected with empty vector (lane 1), hPMCA4b (lane 2) or hPMCA4b-ct125 (lane 3). EP formation was carried out in the presence of 100 μM LaCl_3 and 0.3 μM [γ - ^{32}P]ATP at 4°C for 1 min. The autoradiograph shows 4 μg of phosphorylated microsomes. (B) Ca^{2+} transport at 20 μM Ca^{2+} in the presence and absence of calmodulin (CaM). (C) Ca^{2+} uptake by microsomes prepared from COS-7 cells expressing hPMCA4b (■, □) and hPMCA4b-ct125 (●, ○), in the presence (■, ●) or in the absence (□, ○) of 235 nM calmodulin. Values are means $\pm$ S.D. for three independent experiments.

N-terminus within residues 51–75. An immunoblot using antibody 5F10 is shown in the left panel of Figure 2(A). This demonstrates that the mutant and wild-type pumps were expressed equally in COS-7 cells; expression was approx. 20-fold over the endogenous expression level of PMCA. It has been suggested previously that truncated forms of PMCA4b are more sensitive to proteolysis [22]. Our results clearly demonstrate that no degradation of the PMCA4b-ct125 mutant occurred. The same results were obtained by using either 5F10 (Figure 2A) or JA9 (results not shown) antibodies. The right panel of Figure 2(A) demonstrates that both the wild-type and truncated pumps were phosphorylated equally well when phosphorylation was performed on ice in the presence of lanthanum.

Figure 2(B) shows that the Ca^{2+} transport activity of hPMCA4b-ct125 with or without calmodulin was as high as that of the wild-type protein in the presence of calmodulin. The increase in activity compared with microsomes from cells transfected with empty vector was approx. 15–20-fold, in good agreement with the level of PMCA overexpression shown in Figure 2(A). The addition of calmodulin did not affect the activity of the mutant, whereas it increased the activity of the wild-type pump by approx. 10-fold. It is apparent from the Ca^{2+} curves shown in Figure 2(C) that at low Ca^{2+} concentrations the mutant is more active than the ‘calmodulin-stimulated’ wild-type pump, i.e. its apparent Ca^{2+} affinity is higher. This is consistent with previous findings showing that the affinity of hPMCA4b for calmodulin falls by approx. 5–6 orders of magnitude between 500 and 50 nM Ca^{2+} [28]. This means that the wild-type pump remains inhibited at low Ca^{2+} concentrations because calmodulin does not bind to it under those

conditions. Since the mutant does not need calmodulin for full activity (its activity depends only on substrate Ca^{2+} binding to the catalytic site), it is more active than the wild-type pump at low Ca^{2+} concentrations. These experiments emphasize that removal of the regulatory C-terminus of hPMCA4b downstream of the caspase-3 cleavage site greatly increases basal activity, so that hPMCA4b-ct125 is fully and constitutively active.

These results are in marked contrast with those of Schwab et al. [21]. Although these authors did not measure Ca^{2+} transport, they observed a decrease in single-turnover EP formation after extended incubation of Sf9 cell membranes (expressing hPMCA4b) with caspase-3. Lanthanum is typically included in short 5–30 s incubations with [^{32}P]ATP, as it blocks the dephosphorylation step in the catalytic cycle and thus results in incorporation of 1 mol of ^{32}P per mol of PMCA [27,29]. However, in their experiment Schwab et al. [21] also included lanthanum in the extended caspase digestion reaction. We found that prolonged incubation of microsomes containing either the wild-type or mutant protein in the presence of lanthanum at 37°C decreased the phosphorylated bands dramatically (results not shown). No such effect was observed if the pumps were incubated under similar conditions without lanthanum. Thus the loss of EP shown by Schwab et al. [21] could have been due to a non-specific effect caused by the presence of lanthanum during the extended digestion by caspase-3.

Stable expression and cellular localization of hPMCA4b-ct125 in MDCKII cells

Another important question is whether cleavage by caspase-3 promotes the internalization and possibly further degradation of hPMCA4b, which in fact would also result in loss of activity in the cell. To study further the activity and cellular localization of the caspase cleavage product, MDCKII cells were retrovirally transduced with wild-type or truncated hPMCA4b cDNA. Then MDCKII cell clones were selected that stably expressed the proteins of the predicted size, as determined by immunoblotting. An immunoblot using antibody 5F10 showed that only a small amount of endogenous full-length hPMCA4b could be detected in cells expressing hPMCA4b-ct125, so that the properties of these cells reflected mainly the presence of the mutant (Figure 3A). Again, no degradation product of the expressed hPMCA4b or hPMCA4b-ct125 proteins could be detected by immunostaining with either antibody 5F10 or JA9 (results not shown). Experiments measuring ATP-dependent Ca^{2+} uptake by microsomes of the individual cell clones provided further evidence that the truncated protein was fully functional and independent of calmodulin regulation (Figure 3B).

To determine the intracellular distribution of the truncated protein, MDCKII cells expressing the hPMCA4b variants were grown to confluence. The cells were immunostained and studied by confocal laser scanning fluorescence microscopy (Figure 4). Endogenous Na^+/K^+ -ATPase was used as a basolateral localization marker [30]. In agreement with previous reports [6], we found that wild-type hPMCA4b was localized to the basolateral membrane of MDCKII cells (Figures 4A–4C). This is apparent from the x/z sections, which showed almost exclusively basolateral staining, similar to endogenous Na^+/K^+ -ATPase expression. Examination of six independent clones showed that basolateral sorting was retained for the C-terminally truncated protein (Figures 4D–4F). Untransfected cell clones showed very little detectable PMCA protein, confirming that the level of expression of the endogenous protein was minor compared with the overexpressed hPMCA4b variants.

Figure 3 Characterization of hPMCA4b and hPMCA4b-ct125 expressed in MDCKII cell clones

(A) Immunoblot of portions of 3 μ g of microsomes from MDCKII cells transduced by empty vector (lane 1), hPMCA4b (lane 2, clone 1; lane 3, clone 2) or hPMCA4b-ct125 (lane 4, clone 1; lane 5, clone 8) stained by anti-PMCA antibody 5F10. (B) Ca^{2+} transport activity of microsomes prepared from control and hPMCA4b (clone 2)- or hPMCA4b-ct125 (clone 8)-expressing MDCKII cells measured at 20 μ M Ca^{2+} in the presence or absence of 235 nM calmodulin (CaM). Values are means $\pm$ S.D. for three independent experiments.

Concluding remarks

In summary, the purpose of the present study was to determine whether the primary caspase cleavage fragment of hPMCA4b is active or inactive, and targeted properly to the plasma membrane. Our experiments establish that the initial caspase-3 cleavage product of hPMCA4b is fully and constitutively active. For the first time we generated stable cell clones expressing a constitutively active form of PMCA which lacks the whole regulatory region at its C-terminus and represents the caspase-3 cleavage product of hPMCA4b. In polarized MDCKII cells we demonstrated that the C-terminus is not essential for proper sorting of PMCA4b, since the truncated protein was correctly targeted to the basolateral plasma membrane. Thus we cannot confirm earlier reports of an endoplasmic reticulum retention signal being exposed after removal of the C-terminal tail of hPMCA4b [22]. Also, we cannot confirm that cleavage by caspase-3 inactivates hPMCA4b [21]. Our data, however, are in accordance with previous findings that showed full activation of hPMCA4b upon removal of the regulatory C-terminus [13]. Our results also confirm previous observations that the PDZ-interacting C-terminal sequence is not required for basolateral targeting of PMCA4b [31]. While the experiments described in the present paper do not address the ultimate fate of caspase-cleaved hPMCA4b in the apoptotic cascade, they do provide strong evidence that the initial cleavage of hPMCA4b by caspase produces a constitutively active Ca^{2+} pump, and that this cleavage by itself does not promote internalization in MDCKII epithelial cells. These studies should provide a firm basis for future experiments aimed at elucidating the physiological role of hPMCA4b in apoptosis.

Figure 4 Subcellular localization of hPMCA4b and hPMCA4b-ct125 in a polarized MDCKII monolayer

Double immunofluorescence labelling of PMCA and Na^+/K^+ -ATPase proteins and confocal microscopy were performed as described in the Experimental section. (A–C) hPMCA4b-expressing clone 2; (D–F) hPMCA4b-ct125-expressing clone 8; (G–I) empty vector-transduced cells. Panels (A), (D) and (G) show the localization of hPMCA proteins (green), (B), (E) and (H) show the localization of the endogenous Na^+/K^+ -ATPase (red), whereas the merged images are shown in (C), (F) and (I). An arrow on the right of the merged images indicates the position of the x/z section shown at the bottom of each panel.

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