Deletion of Amino Acid Residues 18–75 Inactivates the Plasma Membrane Ca\(^{2+}\) Pump* 

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A mutant of the plasma membrane Ca\(^{2+}\) pump hPMCA4b(d18–75)(ct120) containing a deletion of the N-terminal amino acid residues 18–75 and lacking the C-terminal 120 amino acid residues was expressed in COS-1 cells. The deletion in the N-terminal region did not significantly affect the level of expression of the Ca\(^{2+}\) pump. Tryptic digestion of the hPMCA4b(d18–75)(ct120) mutant resulted in the appearance of the same fragments obtained by proteolysis of the hPMCA4b(ct120) enzyme, suggesting that deletion of residues 18–75 neither impeded the insertion in the membrane nor extensively affected the folding of the mutant protein. The functional competence of the hPMCA4b(d18–75)(ct120) enzyme was examined by measuring the Ca\(^{2+}\) transport and the Ca\(^{2+}\) ATPase activity of COS-1 cell microsomes expressing the mutant protein. Both tests proved the mutant to be inactive. Under conditions in which hPMCA4b(ct120) becomes phosphorylated, hPMCA4b(d18–75)(ct120) was incapable of reacting with ATP and Ca\(^{2+}\) to form the phosphoryenzym. Taken together these results suggest that the segment of amino acids 18–75 is essential for the activity of the plasma membrane Ca\(^{2+}\) pump.

The Ca\(^{2+}\) pump from plasma membranes (PMCA)\(^1\) is a calmodulin regulated, P\(_2\)-type ATPase (1) responsible for maintaining the intracellular Ca\(^{2+}\) homeostasis in eukaryotic cells by actively extruding Ca\(^{2+}\) to the extracellular space. Four mammalian genes coding for different PMCA isoforms have been identified, each of them producing different mRNAs by alternative splicing processes (2). The PMCA polypeptide would transverse the lipid bilayer about 10 times, and most of the pump, including two major loops and both terminal ends, would be exposed to the cytosol (3). The PMCA contains regions highly conserved in all P-type ATPases and other regions that are distinctive of the PMCA because they are found only in the PMCA isoforms. The regions of the PMCA encompassing the aspartate residue that forms the phosphorylated intermediate (4) and the putative ATP binding site (5) belong to the type of highly conserved regions. In contrast the C-terminal region after the transmembrane segment 10, which is involved in the regulation of the activity of the enzyme by calmodulin (6), and the N-terminal region upstream the first transmembrane segment belong to the regions that are not homologous to that of the other P-type ATPases.

Recently a PMCA mutant called hPMCA4b(ct120) with a deletion of the C-terminal 120 residues including the calmodulin-binding site was expressed in COS-1 cells and found to be fully active, and as expected its activity no longer regulated by calmodulin (7).

We have now investigated the functional relevance of the N-terminal region of the PMCA, which extends from the initial methionine to about the beginning of the first transmembrane segment. With this aim, we constructed a mutant called hPMCA4b(d18–75)(ct120) by removing the nucleotide sequence coding for amino acids 18–75 from the cDNA of hPMCA4b(ct120). The measurements of the activity of the hPMCA4b(d18–75)(ct120) expressed in COS-1 cells indicate that the residues 18–75 are essential for a functional Ca\(^{2+}\) pump.

MATERIALS AND METHODS

Construction of the hPMCA4b Mutant cDNAs and Expression in COS-1 Cells—The construction of the cDNA of mutants hPMCA4b(ct120) and hPMCA4b(d18–75) was described previously, and the latter was called Hinn1 (3, 7). In addition to the deletion of residues 18–75, the presence of a unique restriction site for MluI produced the replacement of serine 17 by threonine in the hPMCA4b(d18–75).

To obtain the hPMCA4b(d18–75)(ct120) mutant, the SphI-DraIII fragment was removed from hPMCA4b(d18–75) and cloned into hPMCA4b(ct120). The wild-type and mutant cDNAs were cloned into the pMM2 vector (7). For protein expression, COS-1 cells (9) were transfected by the DEAE-dextran-chloroquine method (8) and harvested after 48 h. The microsomal fraction was isolated as described previously (7). Protein concentration was estimated by means of the Bio-Rad protein assay, with bovine serum albumin as a standard.

Detection of Expressed Ca\(^{2+}\) Pump Protein—SDS-electrophoresis and immunoblotting were carried out as described previously (10). Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli (12). Vesicles (5–10 μg of protein) were preincubated at 37 °C for 1 h with 50 μl of monoclonal antibody from ascites fluid (dilution, 1:1000). For staining, biotinylated anti-mouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used.

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¶The abbreviations used are: PMCA, Ca\(^{2+}\) pump from plasma membranes; hPMCA4b, human PMCA isoform 4b (the hPMCA4b has also been called hPMCA4CI; Ref. 2).

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Mutant Ca\(^{2+}\) Pump Lacking Residues 18–75

For the topology of the hPMCA4b(d18–75) protein—

**RESULTS**

*Expression of hPMCA4b(ct120) and hPMCA4b(d18–75)(ct120) in COS-1 Cells*—COS-1 cells were transfected with the vector without insert or with either the hPMCA4b(ct120) or the hPMCA4b(d18–75)(ct120) DNAs. Different amounts of membranes from the transfected cells were submitted to SDS gel electrophoresis followed by immunoblotting using antibody 5F10, which recognizes an epitope located between amino acid residues 18–315 of the enzyme. In agreement with previous reports (7), the treatment of COS-1 membranes expressing hPMCA4b(ct120) with 20 \(\mu\)g/ml of trypsin for 30 s resulted in the appearance of a major band of approximately 76 kDa.

Because the N-terminal part of the molecule containing amino acid residues 1–315 is removed early during proteolysis and is not recognized by 5F10 (3) providing the insertion and folding of the enzyme was the same, differences are not expected between the proteolytic pattern of the hPMCA4b(ct120) and hPMCA(d18–75)(ct120). As shown in Fig. 2 this was indeed the case, suggesting that both the hPMCA4b(ct120) and the hPMCA(d18–75)(ct120) proteins exposed the same sites to trypsin and hence produced similar proteolytic fragments containing the epitope for antibody 5F10.

**Activity of the hPMCA4b(d18–75)(ct120) Enzyme**—Results in Table I show that the Ca\(^{2+}\) uptake of microsomes from cells transfected with the hPMCA4b(ct120) DNA was about 12 times higher than that from control microsomes from cells transfected with the empty vector. The Ca\(^{2+}\) transport activity of microsomes expressing the hPMCA4b(d18–75)(ct120) protein was not significantly different from that of the control, indicating that the mutant enzyme was not able to transport Ca\(^{2+}\). As also shown in Table I, the Ca\(^{2+}\) ATPase activity of microsomes from COS-1 cells transfected with the hPMCA4b(d18–75)(ct120) DNA was similar to that of the control, indicating that the hPMCA4b(d18–75)(ct120) was also not capable of hydrolyzing ATP in a Ca\(^{2+}\)-dependent manner.

In control experiments (not shown), the effects of the deletion of amino acid residues 18–75 on the activity of the full-length hPMCA4b was investigated. The hPMCA4b(d18–75) enzyme was also inactive, indicating that the C-terminal regulatory end of the PMCA was not involved in the mechanism leading to inactivation.

**Formation of the Phosphorylated Intermediate**—The first step of the normal Ca\(^{2+}\) pump cycle involves the reaction of the enzyme with Ca\(^{2+}\) and ATP to form a phosphoryzome. The ability of the hPMCA4b(d18–75)(ct120) mutant to form a phosphorylated intermediate was investigated. Fig. 3 shows that two bands corresponding to the endogenous PMCA and sarco/ endoplasmic reticulum Ca\(^{2+}\) pumps were observed when membranes from COS-1 cells transfected with the empty vector were phosphorylated with ATP in the presence of Ca\(^{2+}\) plus La\(^{3+}\). As expected an additional band in the molecular mass region of 120 kDa was observed in membranes containing the

![Immunoblots of microsomes from COS-1 cells transfected with cDNA encoding hPMCA(d18–75)(ct120), hPMCA4b(ct120), or the empty vector pMM2.](image-url)
Mutant Ca$^{2+}$ Pump Lacking Residues 18–75

**TABLE I**

| DNA transfected                              | Ca$^{2+}$ transport | Ca$^{2+}$ ATPase |
|----------------------------------------------|---------------------|-----------------|
| pMM6                                         | 0.14 ± 0.12         | 0.45 ± 0.25     |
| hPMCA4b(ct120)                               | 1.80 ± 0.34         | 6.08 ± 1.08     |
| hPMCA4b(d18–75)(ct120)                       | 0.25 ± 0.12         | 0.33 ± 0.43     |

Early analysis by SDS-polyacrylamide gel electrophoresis of the proteolytic fragments produced by treatment of red cell membranes with trypsin has shown that the protease acts on several specific sites of the PMCA resulting in major peptides of 90, 81, and 76 kDa (15–17). The appearance of the 76-kDa fragment in the SDS-polyacrylamide gel electrophoresis is concomitant with a highly active PMCA. Based on these results and the lack of Ca$^{2+}$ transport by the hPMCA4b(d18–75)(ct120) mutant, it is concluded that the 76-kDa fragment produces a highly active enzyme (7), a mutant with the absence of Ca$^{2+}$ transport. Further, the effective separation of the N-terminal fragment containing the transmembrane domains 1 and 2 from the 76-kDa product in the absence of SDS was not proved.

Recently, the expression of N-terminally and C-terminally truncated PMCA mutants allowed to directly assess the activity of the fragments produced by trypsinolysis. Although truncation of the hPMCA4b generating a C terminus similar to that of the 76-kDa fragment produces a highly active enzyme (7), a mutant starting with the N terminus of the 90-kDa polypeptide (PMCA105) is inactive (18). As was suggested previously (18), the lack of activity of the PMCA105 mutant is probably related to the absence of a large portion of the pump containing the first two transmembrane domains and the region highly conserved in all P-ATPases, which has been called the transducing domain. Results in this paper show that a smaller deletion between the N terminus and the first transmembrane domain, a region previously assumed irrelevant for the function of the enzyme, suffices for inactivation. Studies of mutants with smaller deletions and substitutions of amino acids in the N-terminal region will be needed to obtain a more precise knowledge of the residues involved and their role during Ca$^{2+}$ transport by the PMCA.

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