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Deletion of NH_2- and COOH-terminal sequences destroys function of the Ca^{2+} ATPase of rabbit fast-twitch skeletal muscle sarcoplasmic reticulum

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Deletion mutants of the Ca^{2+} ATPase of rabbit fast-twitch skeletal muscle sarcoplasmic reticulum (SERCA1a) were constructed and expressed in COS-1 cells. The mutants were expressed at levels 7- to 15-fold lower than the wild-type and were inactive. In vitro transcription-translation-insertion experiments showed that deletion of transmembrane sequences M_1 and M_2, but not of M_8, M_9, M_{10} or the NH_2-terminal 30 amino acids inhibited the stable insertion of the enzyme into the membrane. Thus there was no correlation between loss of function and membrane insertion. A signal sequence for membrane insertion may exist in M_1 and M_2.

Ca^{2+} ATPase; Sarcoplasmic reticulum; Mutagenesis

1. INTRODUCTION

The Ca^{2+} ATPase of sarcoplasmic reticulum is a membrane protein of molecular weight 110,000 [1,2]. Primary sequence analysis [2,3] predicts a headpiece, stalk and basepiece structure with ten transmembrane helices. The Ca^{2+} ATPase has been expressed in COS-1 cells [4] and some 250 mutants have been examined for Ca^{2+} transport activity and for their ability to form phosphoenzyme intermediates [5,6]. While 200 of these amino acid substitutions have not resulted in any significant alteration of function, non-functional mutations have been categorized as defective in phosphorylation, Ca^{2+} binding, ATP binding, or conformational changes [6]. The first of these critical amino acid was found at position 111 and the last at position 908, out of 994 amino acids. Of the non critical residues studied, 24 were located upstream of amino acid 111 in the first transmembrane loop and 12 were located downstream of amino acid 908 in the last transmembrane loop. Accordingly, NH_2- or COOH-terminal segments would not appear to be required for the production of a functional protein. To test this possibility, we have constructed deletion mutants at the NH_2- and COOH-termini and measured their enzymatic activity, when expressed in COS-1 cells. We also performed in vitro transcription-translation-insertion experiments on the mutants to examine whether the loss of function we observed in these deletion mutants was related to their ability to insert into microsomes.

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2. EXPERIMENTAL

2.1. *Construction of deletion mutants*

An *Nco*I site was inserted at the initiator methionine in rabbit fast-twitch Ca^{2+} ATPase cDNA [3] by site-directed mutagenesis. Mutant 1 was constructed by removal of an *Nco*I–*Xba*I restriction endonuclease fragment, filling in with Klenow fragment and blunt end ligation. The amino acid sequence at the juncture was Met-Leu-Glu- with removal of cytoplasmic residues 2–32 (Fig. 1). Mutant 2 was constructed by similar removal of an *Xba*I–*Bgl*I fragment, resulting in removal of residues 34–116 comprising predicted transmembrane sequences M_1 and M_2. Mutant 3 was constructed by deletion of a *Bgl*II fragment, followed by religation, resulting in removal of residues 891–973 comprising predicted transmembrane sequences M_8, M_9 and part of M_{10}. Mutant 4 was constructed by blunt end ligation of the cDNA which had been digested with *Bst*E11 and filled in by treatment with Klenow fragment. This resulted in the generation of a TGA stop codon following residue Thr^{906} and removal of residues 907–994 comprising part of predicted transmembrane sequence M_8 and all of M_9 and M_{10}. Mutant 5 was constructed using mutagenesis [7] to convert the codon encoding Lys^{960} to a TAA stop codon, thereby deleting predicted transmembrane sequence M_{10}. Mutant 6 was constructed using mutagenesis to convert the codon encoding Met^{925} to a TAG stop codon, thereby deleting predicted transmembrane sequences M_9 and M_{10}.

The wild type and mutant Ca^{2+} ATPase cDNAs were cloned into the *Eco*RI site of vector p91023(B) [8] for expression in COS-1 cells [18], microsomes were prepared from transfected cells and measurement of Ca^{2+} transport and phosphoenzyme formation were carried out as described previously [4,9,10]. A sandwich enzyme-linked immunosorbent assay was used to quantify the expressed Ca^{2+} ATPase [11], using monoclonal antibody A52 [12].

Ca^{2+} ATPase was solubilized from 100 μg of microsomal protein in $C_{12}E_8$/asolectin (2:1) at a ratio of total protein/$C_{12}E_8$ of 5:1 and immunopurified with A-52 monoclonal antibody (200 μl final volume), as described previously [13]. The Ca^{2+} ATPase–A52–Protein A–

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Sepharose complex was used immediately for measurement of phosphoenzyme formation [13].

For in vitro transcription-translation-insertion experiments, site-directed mutagenesis was used to insert a *NheI* site immediately after the stop codon of Ca^{2+} ATPase and *NheI* linkers were blunt end ligated into the *NcoI* site at the initiator methionine. The wild type and mutant cDNAs were digested with *NheI* and cloned into the *XbaI* site of the plasmid pSP65 (Promega Corporation, Madison WI). This procedure, which effectively removed all of the untranslated 5′ and 3′ regions of the cDNA, was found to be essential for efficient translation. The constructs were linearized with *Hin*dIII and transcribed as described by Melton et al. [14]. The RNA was translated in rabbit reticulocyte lysate with [^{35}S]methionine in the presence and absence of dog pancreatic microsomes, following the protocols from Promega (Translation in vitro, Technical Manual).

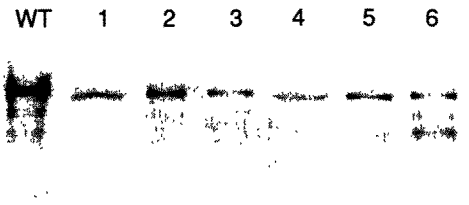

Fig. 2. Expression of Ca^{2+} ATPase deletion mutants. Microsomes were prepared from COS-1 cells transfected with wild type (WT) and mutant Ca^{2+} ATPases (lanes 1–6 for mutants 1–6, respectively). Microsomal proteins were separated on 10% SDS-PAGE (~20 μg/lane) and immunoblotted with monoclonal antibody A52.

3. RESULTS

3.1. *Construction, expression and assay of deletion mutants*

Ca^{2+} ATPase mutations consisting of deletions to either the extreme NH_2-terminus (mutant 1), the first two membrane spanning domains (mutant 2), or various membrane spanning domains at the COOH-terminus (mutants 3–6) were constructed as outlined in Fig. 1. Immunoblot analysis of microsomal proteins from transfected COS-1 cells showed a significant reduction in expression of the deletion mutants, compared to wild type protein and, in some mutants, 'ladder' formation indicating degradation of the expressed protein (Fig. 2). Quantitation of Ca^{2+} ATPase by a sandwich ELISA [11] showed that the deletion mutants were expressed at levels 7- to 15-fold lower than wild type.

Because of the low expression of the deletion mutants and the relatively high endogenous Ca^{2+} ATPase levels in COS-1 cells, it was difficult to measure enzymatic activity without purifying the expressed protein. We have purified it from $C_{12}E_8$ extracts of microsomes by interaction with the A52 monoclonal antibody bound to protein A Sepharose under conditions where at least 50% of wild type ATPase activity is retained [13]. Deletion mutants purified in this manner were not phosphorylated by either ATP or inorganic phosphate (P_i), under conditions in which 20-fold less of the wild type enzyme produced a strong signal corresponding to the phosphoenzyme intermediate band (Fig. 3). Thus deletions as small as 30 amino acids from the NH_2-terminus and 33 amino acids from the COOH-terminus destroyed the earliest steps in the reaction cycle of the Ca^{2+} ATPase.

The deletion mutants may have been inactive and expressed at lower levels because they were not integrated into the membrane in a stable fashion. To examine the ability of the various deletion mutants to insert into membranes, in vitro transcription-translation-insertion experiments were performed (Fig. 4). The wild type Ca^{2+} ATPase and mutants 1, 3, 4, 5, and 6 were found to integrate into the membrane in a fashion resistant to extraction with salt or base under conditions which normally remove all but integral membrane proteins [15]. Analysis of autoradiograms by densitometry showed that an average of 6-fold more of these proteins were pelleted in the presence than in the absence of membranes after extraction at pH 11.5 (compare + and − microsomes, Fig. 4). By contrast, only an average of 1.4-fold more of mutant 2 was pelleted under similar conditions. Thus, mutant 2, lacking the first two transmembrane domains, was the only mutant which interacted weakly with the membranes. Those mutants that were stably associated with the membrane were, nevertheless, expressed at low levels in COS-1 cells.

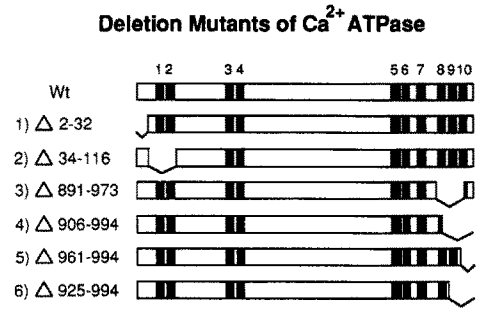

Fig. 1. Construction of deletion mutants of the Ca^{2+} ATPase. The wild type enzyme (Wt) is depicted as a bar, with the ten transmembrane domains shown as black rectangles and labeled 1–10. The deleted amino acids are listed beside each construct, labeled 1–6, and are depicted in the figure by missing parts of the bar.

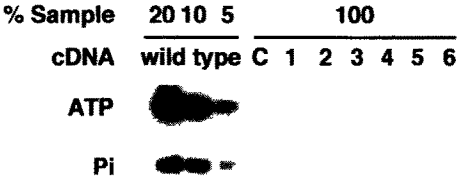

Fig. 3. Phosphorylation of wild type and mutant Ca^{2+} ATPases. Ca^{2+} ATPases were purified and phosphorylated with ATP or inorganic phosphate (Pi). Separation of 20, 10, and 5% of the total wild type sample and 100% of both mutant (lanes 1–6) and control (C) samples was performed on 6% SDS-PAGE in running buffer adjusted to pH 6.3 [17].

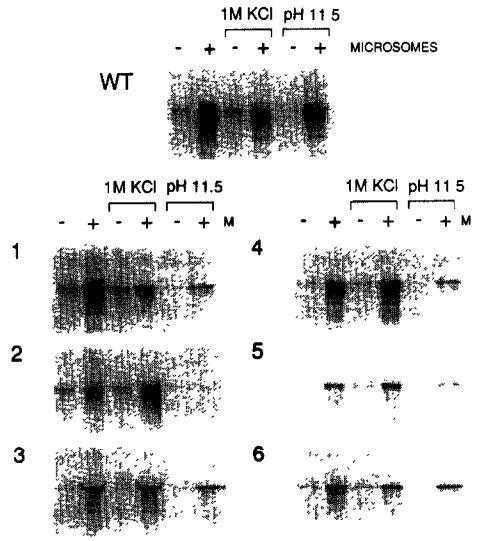

Fig. 4. In vitro insertion of wild type and mutant Ca^{2+} ATPases into dog pancreatic microsomes. Wild type and mutant Ca^{2+} ATPase mRNAs were produced by in vitro transcription with Sp6 RNA polymerase, and the RNA was translated in vitro with [^{35}S]methionine in the presence (+) or absence (−) of dog pancreatic microsomes (M). Samples were either untreated or treated with 1 M KCl or 100 mM Na_2CO_3, pH 11.5, for 10 minutes on ice and recovered by centrifugation in an airfuge operating at 30 psi for 10 minutes. The pellets were separated by 10% SDS-PAGE and subjected to autoradiography.

4. DISCUSSION

The deletion of as little as 3% of the amino acid sequence of the Ca^{2+} ATPase from either the NH_2- or COOH-termini can cause a total loss of enzymatic activity. Although there is no indication from site-directed mutagenesis that specific residues in these sequences are crucial for activity, it is probable that these sequences are required to maintain the three-dimensional structure of the native protein. Examination of the synthetic insertion of the six mutants into membranes showed that deletion of the first transmembrane loop prevented stable membrane integration, while deletion of the cytosolic NH_2-terminal sequence or of transmembrane sequences M_8–M_{10} had no observable effect on membrane insertion. Although loss of activity of mutant 2 could be ascribed to lack of integration into the membrane, this could not be true for mutants 1, 3, 4, 5, or 6.

If five out of the six mutants can be stably integrated into the membrane, then why are they all expressed at low levels in COS-1 cells? Defects in transcription or translation seem unlikely from the in vitro studies. Most probably, the cells have a mechanism of detecting enzymes which are structurally defective and of degrading them through proteolysis. Enzymes defective only in function may escape this surveillance mechanism, since many inactive point mutations are expressed normally in COS-1 cells [4–6]. It is most likely that the deletion mutants do not exist in a native conformation and can be recognized by the cell as defective, regardless of their transmembrane topology.

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