Deletions or Specific Substitutions of a Few Residues in the 
NH2-terminal Region (Ala3 to Thr9) of Sarcoplasmic Reticulum
Ca2+-ATPase Cause Inactivation and Rapid Degradation of the
Enzyme Expressed in COS-1 Cells*

(Received for publication, April 22, 1999, and in revised form, May 20, 1999)

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Amino acid residues in the NH2-terminal region (Glu2–Ala14) of adult fast twitch skeletal muscle sarcoplasmic reticulum Ca2+-ATPase (SERCA1a)1 are deleted or substituted, and the mutants were expressed in COS-1 cells. Deletion of any single residue in the Ala3–Ser6 region or deletion of two or more consecutive residues in the Ala3–Thr9 region caused strongly reduced expression. Substitution mutants A4K, A4D, and H5K also showed very low expression levels. Deletion of any single residue in the Ala3–Ser6 region caused only a small decrease in the specific Ca2+ transport rate/mg of SERCA1a protein. In contrast, other mutants showing low expression levels had greatly reduced specific Ca2+ transport rates. In vitro expression experiments indicated that translation, transcription, and integration into the microsomal membranes were not impaired in the mutants that showed very low expression levels in COS-1 cells. Pulse-chase experiments using [35S]methionine/cysteine labeling of transfected COS-1 cells demonstrated that degradation of the mutants showing low expression levels was substantially faster than that of the wild type. Lactacystin, a specific inhibitor of proteasome, inhibited the degradation of the mutants. His5 in this domain is located very close to the catalytic site. It was previously shown (16) that deletion of most of the residues (Glu2–His32) in the NH2-terminal domain results in greatly reduced expression in COS-1 cells and inactivation of the enzyme. This raises the possibility that the NH2-terminal domain has a region sensitive to the endoplasmic reticulum (ER)-mediated quality control, the machinery of which recognizes and rapidly degrades misfolded proteins (this misfolding can be induced by mutations) and denatured proteins to suppress their cellular expression or accumulation (17, 18). However, the ER-mediated quality control of the Ca2+-ATPase has not yet been reported.

In this study, we have explored the possible roles of much smaller NH2-terminal regions (Glu2–Ala14, especially Ala3–Ser6) than the whole NH2-terminal domain (Met1–Asn399) in cellular expression of SERCA1a and its enzymatic function. We have made 45 mutants of SERCA1a in which residues in the Glu2–Ala14 region have been deleted or substituted, and the mutants have been expressed in COS-1 cells. The results show that deletions or specific substitutions of residues in the Ala3–Thr9 region lead to greatly reduced expression of the mutated SERCA1a proteins and rapid degradation of the expressed SERCA1a proteins. The results further show that residues in the Ala3–Ser6 region are not essential for the Ca2+ transport function. We suggest that the Ala3–Thr9 region is sensitive to the ER-mediated quality control and is thus critical either for correct folding of the SERCA1a protein or stabilization of the correctly folded SERCA1a protein or both.

The Ca2+-ATPase of adult fast-twitch skeletal muscle sarcoplasmic reticulum (SERCA1a)1 is a 994-residue protein (1, 2) that catalyzes Ca2+ transport coupled to ATP hydrolysis across the membrane (3, 4). In the catalytic cycle, this enzyme is activated by Ca2+ binding to the high affinity Ca2+ binding sites, and then the γ-phosphoryl group of ATP is transferred to Asp351 of the enzyme (5–7) to form a phosphoenzyme intermediate (8, 9).

SERCA1a is composed of 10 transmembrane α-helices (M1 to M10) and two main cytoplasmic domains, a small loop (Ala132 to Asp237 between M2 and M3) and a large loop (Asn305 to Asn739 between M4 and M5) (1). In addition, there is a small cytoplasmic NH2-terminal domain (Met1–Asn39), These cytoplasmic domains are connected by α-helical segments (called stalks) to the transmembrane α-helices. The large cytoplasmic loop contains the phosphorylation site and the ATP-binding site. Several residues in the small cytoplasmic loop were shown to play essential roles in the conformational transition of the phosphoenzyme intermediate (10–12). We have recently indicated that Arg258 in this small loop contributes to the catalytic site (13, 14).

The functional role of the NH2-terminal domain is less clear, although our recent chemical modification study (15) has suggested that His5 in this domain is located very close to the catalytic site. It was previously shown (16) that deletion of most of the residues (Glu2–His32) in the NH2-terminal domain results in greatly reduced expression in COS-1 cells and inactivation of the enzyme. This raises the possibility that the NH2-terminal domain has a region sensitive to the endoplasmic reticulum (ER)-mediated quality control, the machinery of which recognizes and rapidly degrades misfolded proteins (this misfolding can be induced by mutations) and denatured proteins to suppress their cellular expression or accumulation (17, 18). However, the ER-mediated quality control of the Ca2+-ATPase has not yet been reported.

EXPERIMENTAL PROCEDURES
Oligonucleotide-directed Mutagenesis and Expression in COS-1 Cells—The methods employed have been described (14). A summary of the methods is as follows. Overlap extension PCR (19) was used to introduce mutations into the rabbit SERCA1a cDNA. The PCR products containing the desired mutation were subcloned into the pT7Blue vector (Novagen, Madison, WI). The mutated fragments were excised and religated back into their original position in the full-length SERCA1a.
SERCA1a was calculated from the initial linear part of the Ca\textsuperscript{2+} transport activity of the expressed SERCA1a was obtained by subtracting the amount of Ca\textsuperscript{2+} transported in the presence of 0.5 μM thapsigargin from that in its absence. The Ca\textsuperscript{2+} transport activity of the expressed SERCA1a was calculated from the initial linear part of the Ca\textsuperscript{2+} transport curve thus obtained. The specific transport rates/mg of SERCA1a protein were calculated from the thapsigargin-sensitive Ca\textsuperscript{2+} transport activity and the amount of the expressed SERCA1a, which was quantified by a sandwich enzyme-linked immunosorbent assay as described by Leberer and Pette (26). In this assay, purified sheep anti-rabbit SERCA1a IgG was used to coat the plates, and monoclonal antibody VE121G9 monoclonal antibody to the rabbit SERCA1a (Affinity Bioreagents, Golden, CO). After incubation with secondary antibody (sheep anti-mouse IgG horseradish peroxidase-conjugated, Amersham Pharmacia Biotech), the bound proteins were probed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Immunoreactivity was quantitated by densitometry.

In the assay, the standard enzyme used was the deoxycholate-purified rabbit SERCA1a that was prepared by the method of Meissner and Fleischer (24) with slight modifications as described previously (25). In addition to Western blotting, quantitation of SERCA1a expression was also obtained by a sandwich enzyme-linked immunosorbent assay as described below.

**Fig. 1. Western blotting of microsomal membranes from COS-1 cells expressing various deletion mutants of SERCA1a.**

The wild-type and deletion mutants of SERCA1a expressed in the microsomal membranes of COS-1 cells were detected by Western blotting as described under “Experimental Procedures.” The deleted amino acid residues are indicated.

**Expression in a Cell-free Transcription/Translation System—PCR mutagenesis was used to insert a HindIII site immediately before the initiation codon of a SacI site introduced from the thapsigargin-sensitive Ca\textsuperscript{2+} transport vector containing no SERCA1a cDNA. Therefore, the Ca\textsuperscript{2+} transport of the expressed SERCA1a was obtained by subtracting the amount of Ca\textsuperscript{2+} transported in the presence of 0.5 μM thapsigargin from that in its absence.**

**Protein concentrations were determined by the method of Lowry et al. (27) with bovine serum albumin as a standard.**

**Miscellaneous Methods—Protein concentrations were determined by the method of Lowry et al. (27) with bovine serum albumin as a standard.**

**RESULTS AND DISCUSSION**

**Effects of Deletions and Substitutions of Residues in the NH\textsubscript{2}-terminal Region of SERCA1a on Expression in COS-1 Cells—Amino acid residues in the NH\textsubscript{2}-terminal Glu\textsuperscript{2}–Ala\textsuperscript{14} region of SERCA1a were deleted or substituted, and the mutants were expressed in COS-1 cells. A typical example of Western blots of the deletion mutants expressed in microsomal membranes is shown in Fig. 1. Visual inspection reveals that expression was only slightly reduced by deletion of 3 residues from Glu\textsuperscript{2} to Ala\textsuperscript{4} but greatly reduced by deletions of 4–13 consecutive residues from Glu\textsuperscript{2} to Ala\textsuperscript{14}. Expression was also...
greatly reduced by deletions of 2–4 consecutive residues from Ser⁶ to Thr⁹.

In addition to the above deletion mutants, 33 mutants were made in which residues in the Glu²–Thr⁹ region were deleted or substituted. The expression levels of the mutants were determined by quantitative densitometry of the proteins visualized with enhanced chemiluminescence and normalized to that of the wild type (Fig. 2). Expression was only partially reduced in the deletion mutants D₂, D₂–3, and D₂–4 but greatly reduced in the mutants with deletions of 4–13 consecutive residues from Glu² to Ala¹⁴, as expected from inspection of Fig. 1. Expression of the mutants with deletions of 2–4 consecutive residues in the Ala³–Thr⁹ region was also greatly reduced. Strikingly, expression of the mutants with deletions of any single residue in the Ala³–Ser⁶ region was markedly reduced. Expression was greatly reduced in the substitution mutants A₄K, A₄D, and H₅K but not significantly or only partially reduced in other substitution mutants tested. These results indicate that deletions of 1 or more residues in the Ala³–Thr⁹ region or specific substitutions of Ala⁴ with lysine and aspartic acid or His⁵ with lysine result in strongly reduced expression in COS-1 cells.

Effects of Deletions and Substitutions of Residues in the NH₂-terminal Region of SERCA₁a on the Ca²⁺ Transport Rate—The specific Ca²⁺ transport rates/mg of SERCA₁a protein were determined with the mutants in which residues in the NH₂-terminal Glu²–Ser⁸ region were deleted or substituted, and the rates were normalized to that of the wild-type SERCA₁a (Fig. 3). Deletion of any single residue in the Ala³–Ser⁶ region caused only a small decrease in the specific Ca²⁺ transport rate. This indicates that these single-residue deletions have only small effects on the enzyme structure essential for Ca²⁺ transport function. The specific Ca²⁺ transport rates in the substitution mutants A₄D, A₄L, H₅D, and S₆L were not significantly different from that of the wild type. These results show that the amino acid residues in the Ala³–Ser⁶ region are not essential for Ca²⁺ transport function. Previously, Skerjanc et al. (16) reported that there is no indication from site-directed mutagenesis that specific residues in the Glu²–His₃² region are crucial for enzymatic activity. This is consistent with our above conclusion.

On the other hand, when 2–4 consecutive residues in the Glu²–Ser⁸ region were deleted (D₂–5, D₃–4, D₄–5, D₅–6, and D₆–8), the specific Ca²⁺ transport rates were greatly reduced. The transport rates were also greatly reduced in the substitution mutants A₄K, A₄D, and H₅K. These results indicate that deletions of 2 or more residues in the Glu²–Ser⁸ region, or specific substitutions of Ala⁴ with lysine and aspartic acid or His⁵ with lysine, induce structural changes that lead to inactivation of the enzyme. This is in harmony with our previous results from chemical modification of His⁵ (15) suggesting that His⁵ is located very close to the catalytic site.

In Vitro Expression of Mutant SERCA₁a in Pancreatic Microsomal Membranes—In vitro expression experiments were performed with a cell-free transcription/translation system containing pancreatic microsomal membranes in the presence of [³⁵S]methionine (Fig. 4). The wild-type SERCA₁a or five mutants (Δ₂–5, Δ₄–5, Δ₅–6, Δ₆–8, and A₄K) showing very low expression levels in COS-1 cells (see Fig. 2) were expressed in this system. The membrane fractions isolated were either untreated or treated with salt or base under conditions...
in which all but integral proteins are usually removed (29), and the samples were subjected to SDS-polyacrylamide gel electrophoresis. The digital autoradiogram of the gel showed a single major band for each sample (Fig. 4,
left panel) at the position of the rabbit SERCA1a (Fig. 4,
arrows). These major bands were identified as the expressed SERCA1a protein by Western blotting with the monoclonal anti-rabbit SERCA1a antibody (Fig. 4,
right panel). The digital autoradiogram and Western blot showed that the expression levels of the mutants were similar to or even higher than those of the wild type SERCA1a and that all the mutants tested and the wild type were integrated into the membrane in a fashion resistant to extraction with salt or base. These results indicate that transcription, translation, and integration into the microsomal membranes are not impaired in these mutants. This raised the possibility that these mutants are degraded rapidly in COS-1 cells, although the interference of the mutations with the membrane assembly of the expressed SERCA1a in COS-1 cells is also possible because such interference was previously demonstrated by Zhang et al. (30) with the mutations in the SERCA1a segment from the phosphorylation site (Asp351) to the transmembrane helix M4. Thus, we examined the degradation of the mutants in COS-1 cells by pulse-chase experiments.

Degradation of Mutant SERCA1a in COS-1 Cells—COS-1 cells expressing the wild-type or mutant SERCA1a were pulse-labeled with [35S]methionine/cysteine, chased, and then lysed. The SERCA1a in the lysate was immunoprecipitated. Lysate volumes for immunoprecipitation were normalized by trichloroacetic acid-precipitable radioactivity. The radioactivity of the

![Fig. 3](image3.png)

**Fig. 3.** The rates of Ca^{2+} transport catalyzed by deletion and substitution mutants of SERCA1a in microsomal membranes from COS-1 cells. The specific Ca^{2+} transport rates/mg of SERCA1a protein of various deletion and substitution mutants of SERCA1a in the microsomal membranes were obtained as described under “Experimental Procedures” and normalized to that of the wild-type SERCA1a (100%). The values presented are the mean ± S.D. of five independent transfections. The specific Ca^{2+} transport rate of the wild-type SERCA1a was 8–11 μmol of Ca^{2+}/min/mg of SERCA1a protein.

![Fig. 4](image4.png)

**Fig. 4.** In vitro expression of mutant SERCA1a in pancreatic microsomal membranes. The wild-type or mutant SERCA1a was expressed in a cell-free transcription/translation system containing pancreatic microsomal membranes in the presence of [35S]methionine as described under “Experimental Procedures.” Membrane fractions isolated were either untreated or treated with 1 M KCl or 100 mM Na2CO3 (pH 11.5) and then subjected to SDS-polyacrylamide gel electrophoresis. The left panel is a digital autoradiogram of [35S]methionine-labeled proteins; the right panel is a Western blot of a gel loaded with the same samples as in the left panel. Control, expression was performed with pSP64 poly(A) vector harboring no SERCA1a cDNA. SR Ca^{2+}-ATPase, the deoxycholate-purified rabbit SERCA1a (25). The positions of the rabbit SERCA1a are indicated by arrows.
SERCA1a thus obtained was quantitated by SDS-polyacrylamide gel electrophoresis and digital autoradiography (Fig. 5). Relative amounts of the radiolabeled wild-type SERCA1a increased during the chase period. This shows that degradation of the wild-type SERCA1a was slower than the decrease in the total trichloroacetic acid-precipitable radioactivity. Degradation was not affected by the H5D substitution, which had no effect on the expression level in COS-1 cells (see Fig. 2) and on the specific Ca\textsuperscript{2+} transport rate (see Fig. 3). In contrast, degradation was accelerated moderately in the single-residue deletion mutants Δ3, Δ4, Δ5, and Δ6, in which expression in COS-1 cells was reduced substantially (see Fig. 2) but the specific Ca\textsuperscript{2+} transport rate was reduced only partially (see Fig. 3). Degradation was more strongly accelerated in the 2-residue deletion mutants Δ3–4, Δ4–5, and Δ5–6 and the substitution mutants in which both the expression levels in COS-1 cells (see Fig. 2) and the specific Ca\textsuperscript{2+} transport rates (see Fig. 3) were reduced greatly. Degradation was most strongly accelerated in the 4-residue deletion mutant Δ2–5, in which expression in COS-1 cells was at the lowest level (see Fig. 2) and the specific Ca\textsuperscript{2+} transport rate was abolished almost completely (see Fig. 3). These results indicate that the reduced expression of these mutants in COS-1 cells was due to accelerated intracellular degradation of the mutants. The results further suggest that substantial acceleration of degradation and strong suppression of cellular expression of the mutants probably can be induced even by small structural changes that have only small effects on the specific Ca\textsuperscript{2+} transport rate as shown with the single-residue deletion mutants.

It is well documented that the single-residue deletion of phenylalanine (ΔF508) from a cytoplasmic portion of cystic fibrosis transmembrane conductance regulator (CFTR), which is a 1480-residue protein containing 12 putative transmembrane segments, induces its rapid ER quality control-mediated degradation and leads to greatly reduced plasma membrane expression but does not severely impair the function of CFTR (31). This situation closely resembles our present results with the single-residue deletion mutants. This prompted us to explore the possibility that structural changes induced by deletions or substitutions in the Glu\textsuperscript{2–Ser\textsuperscript{6}} region of SERCA1a are
recognized by the ER quality control machinery.

The effect of lactacystin, a specific proteasome inhibitor, on degradation of the mutants was examined (Fig. 6). The presence of 10 μM lactacystin in the medium throughout the pulse and chase periods resulted in a substantially reduced rate of degradation of the single-residue deletion mutant Δ3. This indicates that proteasome is involved in the Δ3 degradation is by the ER quality control machinery as the mutant ΔF508 of CFTR. However, lactacystin did not affect the degradation of the mutants Δ2–5, Δ4–5, Δ5–6, and H5K, whose degradation was much more rapid than that of the single-residue deletion mutants (see Fig. 5). This finding suggests that a lactacystin-insensitive protease(s), rather than proteasome, is involved in the very rapid degradation of these mutants. Unexpectedly, degradation of the wild type was accelerated by addition of lactacystin. The reason for this acceleration remains obscure.

No degradation intermediates were detected by immunoprecipitation analysis using polyclonal anti-rabbit SERCA1a antibody in the pulse-chase experiments (data not shown), in agreement with the previously reported findings that no degradation intermediates were detected in the ER quality control of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (32) and the mutated ATP-binding cassette transporter Pdr5 (33). This suggests that the rate-limiting step in the degradation of the mutants occurs before proteolysis by proteasome or other proteases. It is likely that this rate-limiting step (possibly unfolding of the protein) is more strongly accelerated by deletions of 2 or more residues in the Glu2–Ser6 region or by the A4D or H5K substitution than by single-residue deletions in the Ala3–Ser6 region.

The present results indicate that the NH2-terminal region (Ala3–Thr9) of SERCA1a is very sensitive to the ER quality control, the machinery of which recognizes misfolded or denatured proteins and rapidly degrades these abnormal proteins (17, 18). Therefore, it is very likely that this region is critical for either correct folding of the SERCA1a protein or stabilization of the correctly folded SERCA1a protein or both. Single mutations in other sequence segments in the SERCA1a were previously reported to have effects similar to those reported in this study. Zhang et al. (30) showed that mutation of Ala231 to Arg yields very low protein levels in COS-1 cells, whereas transcription is normal. Xu et al. (34) reported that mutation of Phe256 to Glu reduces expression greatly but not enzyme activity.

The NH2-terminal region (Ala3–Thr9) of SERCA1a shares virtually no homology with the NH2-terminal domains of plasma membrane Ca2+-ATPase (35), Na+,K+-ATPase (36, 37), or H+,K+-ATPase (38, 39). It was previously shown that deletion of the NH2-terminal 18–75 residues from the plasma membrane Ca2+-ATPase (40) or deletion of the NH2-terminal 1–32 residues from the Na+,K+-ATPase (41) does not inhibit cellular expression of the protein. It is possible that the role of the NH2-terminal region described in this paper is specific to the family of sarco(endo)plasmic reticulum Ca2+-ATPases, because the NH2-terminal domains of the Ca2+-ATPases in this family have considerably high homology to each other (42).