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Mutation of aspartic acid-351, lysine-352, and lysine-515 alters the Ca^{2+} transport activity of the Ca^{2+}-ATPase expressed in COS-1 cells

(sarcoplasmic reticulum/site-specific mutation)

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ABSTRACT **Full-length cDNAs encoding neonatal and adult isoforms of the Ca^{2+}-ATPase of rabbit fast-twitch skeletal muscle sarcoplasmic reticulum were expressed transiently in COS-1 cells. The microsomal fraction isolated from transfected COS-1 cells contained immunoreactive Ca^{2+}-ATPase and catalyzed Ca^{2+} transport at rates at least 15-fold above controls. No differences were observed in either the rates or Ca^{2+} dependency of Ca^{2+} transport catalyzed by the two isoforms. Aspartic acid-351, the site of formation of the catalytic acyl phosphate in the enzyme, was mutated to asparagine, glutamic acid, serine, threonine, histidine, or alanine. In every case, Ca^{2+} transport activity and Ca^{2+}-dependent phosphorylation were eliminated. Ca^{2+} transport was also eliminated by mutation of lysine-352 to arginine, glutamine, or glutamic acid or by mutation of Asp^{351}-Lys^{352} to Lys^{351}-Asp^{352}. Mutation of lysine-515, the site of fluorescein isothiocyanate modification in the enzyme, resulted in diminished Ca^{2+} transport activity as follows: arginine, 60%; glutamine, 25%; glutamic acid, 5%. These results demonstrate the absolute requirement of acylphosphate formation for the Ca^{2+} transport function and define a residue important for ATP binding. They also demonstrate the feasibility of a thorough analysis of active sites in the Ca^{2+}-ATPase by expression and site-specific mutagenesis.**

The Ca^{2+}-ATPase of the sarcoplasmic reticulum plays a key role in regulation of muscle function. By pumping Ca^{2+} from the sarcoplasm to luminal spaces in the organelle, it is able to lower sarcoplasmic Ca^{2+} concentration to the range of 100 nM, thereby inducing and maintaining muscle relaxation (1). The enzyme has been studied extensively and a great deal is known about its reaction kinetics (2) and structure (3). cDNAs encoding two forms of the enzyme have been cloned (4–6) and hypotheses concerning structure (4) and function (5) have been presented on the basis of the primary sequences and predicted folding patterns of these two enzymes.

The availability of full-length clones of the Ca^{2+}-ATPase has permitted us to express the proteins in functional form and to use site-directed mutagenesis as a tool to evaluate hypotheses concerning structure and function. In this paper, we describe the results of mutation of aspartic acid-351, the site of catalytic phosphorylation in the enzyme (7), and the adjacent residue lysine-352. We also describe the results of mutation of lysine-515, the residue modified by fluorescein isothiocyanate and believed to be involved in ATP binding (8, 9).

MATERIAL AND METHODS

Constructs for Expression and Mutation. Our expression system is composed of the full-length fast-twitch Ca^{2+}-ATPase cDNA (5) ligated into the vector p91023(B) (10) and transfected into COS-1 cells (11). Our original neonatal Ca^{2+}-ATPase cDNA clone (5) was isolated in the vector pCDX (12). The sequence used for expression extended from the *Pst* I site at the 5′ end of the cDNA, formed in part from the GC tails used in its insertion into the vector, to the *Xho* I site in the vector ≈30 bases downstream of the 3′ end of the cDNA insert. *Eco*RI linkers were attached to both free ends so that the entire ATPase cDNA inserts could be shuttled from vector to vector by ligation into an appropriate *Eco*RI site. To create a construct from which fragments of the Ca^{2+}-ATPase could be excised for mutation and returned readily, we excised a 445-base-pair (bp) *Pvu* II/*Pvu* II fragment from the Bluescript vector (Stratagene, La Jolla, CA), thereby removing the T3 and T7 promoters and the multiple cloning sites and creating a small plasmid (≈2500 bp) with relatively few restriction endonuclease cleavage sites. We attached *Eco*RI linkers to the half *Pvu* II sites in the truncated plasmid and ligated it with the full-length Ca^{2+}-ATPase cDNA insert to form the plasmid pBSfN (neonatal). The plasmid pBSfA (adult) was constructed by exchanging residues 2694–3165 (5) in pBSfN with residues 2694–3207 in the adult cDNA clone (6) in the form of *Nco* I restriction endonuclease fragments.

Fragments to be mutated were removed from the pBSf plasmids and ligated into the polylinker in the intact Bluescript vector for site-specific mutagenesis by the method of Kunkel (13). All manipulations of DNA were carried out by standard methods (14, 15). Synthetic oligonucleotides, 17–22 bases long and containing the single mutated base near the center, or with multiple mismatches near the center and flanked by 8 bases of complementary sequence, were hybridized to the fragment of ATPase cDNA inserted in the single-stranded template to begin mutagenesis. Dideoxy sequencing (16) was carried out to confirm that each sequence was mutated. After sequencing, the appropriate point-mutated fragments were ligated back into their original positions in the pBSf plasmids. The entire ATPase cDNA was then excised and cloned into the *Eco*RI site of p91023(B) for expression in mammalian cells.

Cell Culture and DNA Transfection. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 0.1 mM α-MEM nonessential amino acids, 4 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml, and 10% fetal calf serum under 5% CO_2/95% air at 37°C. DNA transfection was carried out by the DEAE dextran-chloroquine shock method (17, 18) with 25 μg of cesium chloride gradient-purified DNA and 1.5 mg of DEAE dextran per 10-cm Petri dish. Cells were then incubated for 3 hr at 37°C in 6 ml of DMEM containing 300 μg of chloroquine, washed, and cultured in DMEM for 48 or

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72 hr. Control cells were treated in the same way with vector DNA or with no added DNA.

RNase Protection Assay. Total RNA was prepared from control and transfected cells as described (19) and 10 μg was used in each RNase protection assay. The anti-sense radiolabeled RNA probe was synthesized with T7 RNA polymerase from a 317-bp fragment of the 3′ region of adult fast-twitch muscle Ca^{2+}-ATPase cDNA [residues 2915–3232 (6)], which had been cloned into the vector pTZ (Pharmacia). RNase protection assays were carried out as described (6).

Isolation of a Microsomal Fraction. For isolation of a microsomal fraction (20, 21), cells from five 10-cm Petri dishes were washed twice with 5 ml of a solution of 0.137 M NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4 (PBS), harvested in a solution of 5 mM EDTA in PBS and washed with 5 ml of PBS. The cells were swollen at 0°C for 10 min in 2 ml of a hypotonic solution of 10 mM Tris·HCl, pH 7.5/0.5 mM $MgCl_2$, and then phenylmethylsulfonyl fluoride and Trasylol were added to 0.1 mM and 100 units/ml, respectively. The cells were homogenized with 30 strokes in a glass Dounce homogenizer and the homogenate was diluted with an equal volume of a solution of 0.5 M sucrose/6 mM 2-mercaptoethanol/40 μM $CaCl_2$/300 mM KCl/10 mM Tris·HCl, pH 7.5. The suspension was centrifuged at 10,000 × *g* for 20 min to pellet nuclei and mitochondria. The supernatant was made 0.6 M in KCl by the addition of 0.9 ml of a 2.5 M solution. The suspension was centrifuged at 100,000 × *g* for 60 min to sediment the microsomal fraction. The pellet was suspended in a solution containing 0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol, 20 μM $CaCl_2$, 10 mM Tris·HCl (pH 7.5), and centrifuged again at 100,000 × *g* for 60 min. The final pellet, containing ≈100 μg of protein, was suspended in the same solution at a protein concentration of 1 mg/ml.

Ca^{2+} Transport Assay. Ca^{2+} transport activity was assayed in a reaction mixture containing 20 mM Mops-KOH (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.45 mM $CaCl_2$ (containing ^{45}Ca at a specific activity of 10^6 cpm/μmol), 0.5 mM EGTA, and 5 mM potassium oxalate. The uptake reaction was initiated by the addition of 10 μg of protein to 1 ml of reaction mixture at room temperature. At different time periods, 0.15-ml samples were filtered through a 0.3-μm Millipore filter and washed with 10 ml of 0.15 M KCl. Radioactivity on the filters was measured by liquid scintillation counting. For the measurement of Ca^{2+} ion dependency, free Ca^{2+} concentrations were calculated by the computer program of Fabiato and Fabiato (22). For studies of ATP concentration dependency, an ATP regenerating system consisting of 2.5 mM phospho*enol*pyruvate and 50 μg of pyruvate kinase per ml was used.

Phosphorylated Intermediate. Microsomal protein (5 μg) was added to 0.1 ml of a solution of 20 mM Mops, pH 6.8/100 mM KCl/5 mM $MgCl_2$/0.5 mM EGTA in the presence or absence of 0.5 mM $CaCl_2$. The reaction, at ice temperature, was started by the addition of 5 μM ATP (10^6 cpm/nmol) and stopped after 5 sec by the addition of 0.6 ml of a mixture of 5% trichloroacetic acid and 5 mM potassium phosphate. Incorporation of ^{32}P was determined either by collecting the protein on a filter for scintillation counting or by separating the protein in acidic $NaDodSO_4$/polyacrylamide gels for autoradiography (23).

Miscellaneous Assays. Protein concentration was determined by dye binding using bovine serum albumin as a standard (24). $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out by the method of Laemmli (25). Immunoblotting was carried out by the method of Towbin *et al.* (26). The monoclonal antibody against the fast-twitch Ca^{2+}-ATPase was A52 described by Zubrzycka-Gaarn *et al.* (27). The antibody–antigen complex was detected with an alkaline phosphatase-conjugated second antibody (Promega Biotec, Madison, WI).

RESULTS

Expression of the Fast-Twitch Ca^{2+}-ATPase. Transcription of the fast twitch Ca^{2+}-ATPase cDNA in transfected COS-1 cells was detected by an RNase protection assay using antisense RNA synthesized from a 317-bp fragment at the 3′ end of the adult form of the fast-twitch Ca^{2+}-ATPase (6). This probe sequence protects 317 bp in the adult isoform, but since it contains a 42-bp exon, which is spliced out of neonatal cDNA, it will protect only 65- and 210-bp fragments in the neonatal isoform. Fig. 1*A* illustrates that protection of these two fragments was observed with RNA extracted from COS-1 cells transfected with cDNA encoding the neonatal isoform, but no protection was observed with control cells. Since the RNase protection assay required complete homology between mRNA and the radiolabeled antisense RNA probe, it was clear that transfected, neonatal, fast-twitch Ca^{2+}-ATPase cDNA was transcribed in the transfected COS-1 cells.

Expression of Ca^{2+}-ATPase protein was detected by using an immunoblot procedure with monoclonal antibody A52 (27). In Fig. 1*B*, we demonstrate that the fast-twitch Ca^{2+}-ATPase is expressed in transfected COS-1 cells but not in control cells. Fig. 1*B* also shows that the Ca^{2+}-ATPase was concentrated in the microsomal fraction after cell fractionation. Densitometry of the stained bands compared to authentic fast-twitch ATPase protein indicated that the expressed ATPase concentration was ≈0.5 μg per 10 μg of microsomal protein.

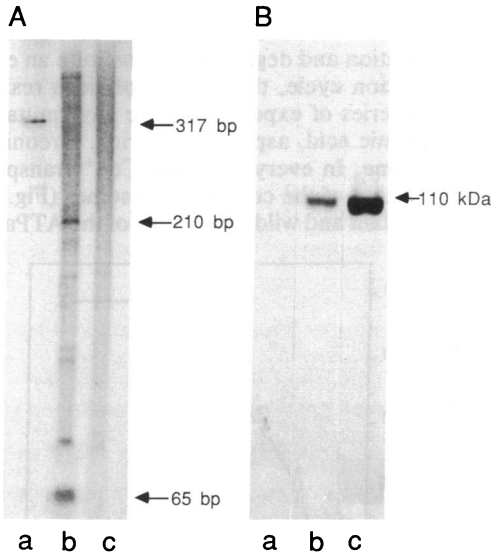

FIG. 1. Analysis of transcription and translation of the Ca^{2+}-ATPase cDNA in transfected COS-1 cells. (*A*) A 317-base RNA probe (lane a) was synthesized from the 3′ end of the adult fast-twitch Ca^{2+}-ATPase cDNA (6). This probe was hybridized with poly(A)$^+$ RNA isolated from COS-1 cells transfected with neonatal Ca^{2+}-ATPase cDNA (lane b) or from nontransfected cells (lane c). The hybrids were then digested with RNases A and T1 (6), separated on polyacrylamide gels, and subjected to autoradiography. Protection of 210- and 65-base fragments is characteristic of a transcript of the neonatal cDNA. (*B*) Immunoblotting with antibody A52 (27) of a 110-kDa protein in the homogenate (lane a) of nontransfected COS-1 cells and the homogenate (lane b) and microsomal fraction (lane c) from COS-1 cells transfected with the adult Ca^{2+}-ATPase cDNA. Fifty micrograms of protein was applied to the gels for homogenate fractions and 10 μg of protein was applied for the transfected microsomal fraction.

Ca^{2+} Transport Activity. The presence of a functional Ca^{2+}-ATPase in microsomal fractions of transfected cells was demonstrated by Ca^{2+} transport assays. The Ca^{2+} transport activity of the microsomal fraction isolated from transfected cells was at least 15 times higher than the Ca^{2+} transport activity of control microsomes (Fig. 2). In our experiments, Ca^{2+} uptake rates were usually linear for at least 20 min and were not inhibited by 5 mM sodium azide. No Ca^{2+} uptake was observed when the ionophore A23187 was present in the assay.

By varying Ca^{2+} concentrations and measuring the concentration required for half-maximal activity of the Ca^{2+} pump, it was possible to estimate Ca^{2+} affinities for the expressed enzymes. The same principle has been found to apply for measurement of ATP affinity (data not shown). The affinity of the expressed enzyme for Ca^{2+} was comparable to that of intact sarcoplasmic reticulum (2), with a half-maximal Ca^{2+} uptake rate occurring at 2×10^{-7} M Ca^{2+} (Fig. 3).

Comparison of Ca^{2+} Transport Activities of Adult and Neonatal Isoforms. The adult form of the rabbit fast-twitch Ca^{2+}-ATPase protein ends in a glycine residue (28). Studies of cDNA sequences indicate that two alternatively spliced transcripts exist in neonatal muscle with one form (adult) encoding a carboxyl-terminal glycine and the other (neonatal) encoding a highly charged carboxyl-terminal sequence -Asp-Pro-Glu-Asp-Glu-Glu-Arg-Arg-Lys (5, 6). It was of interest to determine whether these alternatively spliced forms of the enzyme differed in their Ca^{2+} transport activities. The data in Fig. 2 show that the adult and neonatal isoforms had virtually identical Ca^{2+} transport activity, while the data in Fig. 3 show that their affinities for Ca^{2+} were identical.

Mutation of Aspartic Acid-351 and Lysine-352. The site of catalytic phosphorylation in the Ca^{2+}-ATPase has been identified as aspartic acid-351 (7). Since it is thought that acyl phosphate formation and degradation constitute an essential part of the reaction cycle, this was an obvious residue for mutation. In a series of experiments, we have mutated this residue to glutamic acid, asparagine, serine, threonine, histidine, and alanine. In every case, the Ca^{2+} transport rate was reduced to that of the control microsomes (Fig. 4*A*). In all cases, the mutant and wild-type forms of the ATPase were

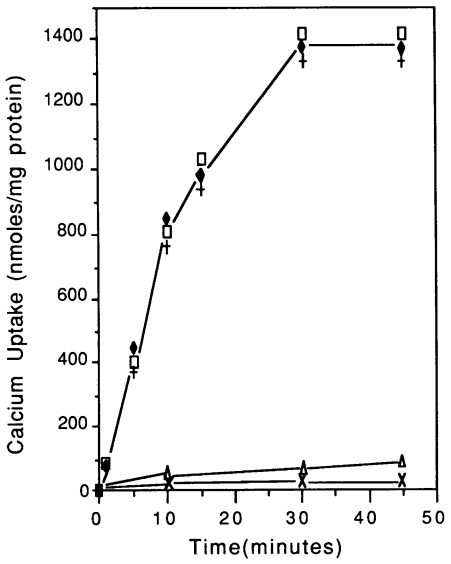

FIG. 2. Ca^{2+} transport by microsomes isolated from nontransfected and transfected COS-1 cells. □, Adult Ca^{2+}-ATPase cDNA; ◆, neonatal Ca^{2+}-ATPase cDNA; + and ×, adult Ca^{2+}-ATPase cDNA, reaction carried out in the presence of 5 mM sodium azide (+) or in the presence of 2 μM A23187 (×); △, nontransfected cells.

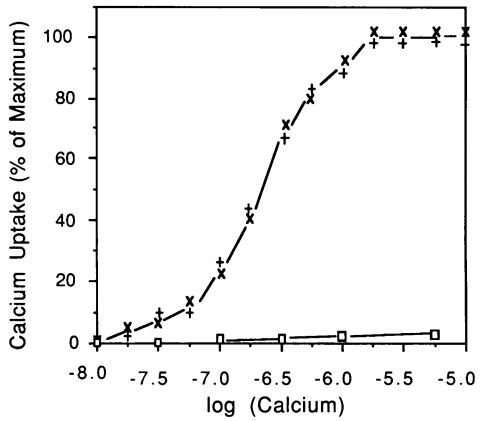

FIG. 3. Ca^{2+} dependency of Ca^{2+} transport catalyzed by the expressed adult and neonatal Ca^{2+}-ATPases. Ca^{2+} transport assays were carried out for a fixed time of 15 min in the presence of various concentrations of free Ca^{2+}. Maximum Ca^{2+} accumulation (100%) was 1000 nmol per mg of protein at 15 min. The rates of Ca^{2+} uptake provide an estimate of the affinity of the expressed Ca^{2+}-ATPase for Ca^{2+}. ×, Adult Ca^{2+}-ATPase cDNA; +, neonatal Ca^{2+}-ATPase cDNA; □, no added cDNA.

expressed to approximately the same extent as judged by immunoblotting (Fig. 4*B*).

The availability of Ca^{2+}-ATPase preparations with alternative residues at position 351 allowed us to confirm that this residue was the site of catalytic phosphorylation. When we

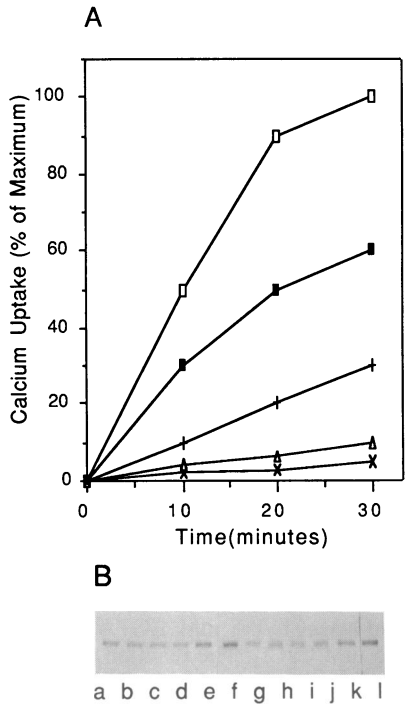

FIG. 4. (*A*) Ca^{2+} uptake by microsomes isolated from COS-1 cells transfected with adult Ca^{2+}-ATPase cDNA (□); or lysine-515 to arginine (■), glutamine (+), or glutamic acid (△); or no cDNA or cDNAs mutated as follows (×): aspartic acid-351 to asparagine, glutamic acid, serine, or alanine; lysine-352 to arginine, glutamine, or glutamic acid; Asp^{351}-Lys^{352} to Lys^{351}-Asp^{352}. The control Ca^{2+} transport rate was 75 nmol of Ca^{2+} per mg of protein per min, while the maximum accumulation was 1300 nmol of Ca^{2+} per mg of protein at 30 min. (*B*) Immunoblotting of the various mutant ATPases expressed in COS-1 cell microsomes and assayed for Ca^{2+} transport in *A*. Lanes: a, wild type; b–d, lysine-515 to arginine, glutamine, and glutamic acid, respectively; e–i, aspartic acid-351 to glutamic acid, asparagine, threonine, serine, and alanine, respectively; j–l, lysine-352 to arginine, glutamine, and glutamic acid, respectively.

Table 1. Phosphorylation of microsomes from transfected COS-1 cells

Transfected cDNA	Phosphorylation, pmol per mg of protein	
	+ Ca^{2+}	+ EGTA
No cDNA (control)	58	61
Wild type	217	74
Mutant Asp^{351} to Glu^{351}	51	55
Mutant Asp^{351} to Ala^{351}	55	57

phosphorylated the membrane preparation with [γ-^{32}P]ATP, Ca^{2+}-dependent incorporation of ^{32}P was 217 pmol per mg of protein when the cells were transfected with the wild-type ATPase cDNA (Table 1). In nontransfected cells or in cells transfected with ATPases mutant at aspartic acid-351, the incorporation of ^{32}P was 51–58 pmol per mg of protein, values very similar to those obtained in the presence of 5 mM EGTA. When phosphorylated microsomal preparations from COS-1 cells transfected with wild-type and mutant cDNAs were separated on acidic polyacrylamide gels and subjected to autoradiography, a ^{32}P-labeled band of 110 kDa was observed with wild-type cDNA but not with any of the mutant cDNAs (Fig. 5).

Although aspartic acid-351 was identified as the phosphorylation site (7), it is significant that all eukaryotic cation transport ATPases so far sequenced have the conserved sequence -Cys-Ser-Asp*-Lys-Thr-Gly-Thr-Leu-Thr- at the phosphorylation site (N. M. Green, W. R. Taylor, and D.H.M., unpublished review). Accordingly, it was of interest to mutate other residues around aspartic acid-351. Data presented in Fig. 4 demonstrate that mutation of lysine-352 to arginine, glutamic acid, or glutamine, or exchange of Asp^{351}-Lys^{352} for Lys^{351}-Asp^{352} all eliminated the Ca^{2+}-transport function of the enzyme.

Mutations at Lysine-515 (Fluorescein Isothiocyanate Binding Site). Lysine-515 has been implicated in the ATP-binding site of the Ca^{2+}-ATPase because modification of this residue by fluorescein isothiocyanate results in inhibition of ATP binding and ATP hydrolysis, although the enzyme is still capable of hydrolyzing acetyl phosphate (8). We mutated lysine-515 to arginine, glutamine, and glutamic acid and measured Ca^{2+} transport activity in the expressed products. The mutation to glutamic acid was virtually inactive, the mutation to glutamine was reduced to 25% of control activity, and the mutation to arginine was reduced to 60% of wild-type Ca^{2+} transport activity (Fig. 4).

DISCUSSION

Expression of the Ca^{2+}-ATPase. Progress in recombinant DNA technology has made it feasible to consider the expression and mutation of full-length cDNAs encoding a variety of proteins (29–31). Since our interests lie in the structure and function of the Ca^{2+}-ATPase, consideration had to be given to the appropriate conditions for the expression and mutation of this protein. First, the Ca^{2+}-ATPase is a membrane protein entirely dependent on phospholipid for activity. Second, the protein is normally located in internal membranes. Third, the function of the protein can most readily be measured in a Ca^{2+}-transport assay, which requires rather large amounts of expressed protein. Accordingly, we decided to express the Ca^{2+}-ATPase in mammalian cells so that it could be incorporated into the endoplasmic reticulum of these cells in association with mammalian phospholipids. We chose the vector p91023(B) (10) because of its high expression efficiency. We chose the COS-1 cell line (11), a monkey kidney cell line transformed by an origin-defective mutant of simian virus 40, because it expresses the large tumor antigen, which will promote a high copy number of plasmids containing the simian virus 40 origin of replication. With this system, we have achieved the expression of a highly active fast-twitch Ca^{2+}-ATPase with the same characteristics as those found in the sarcoplasmic reticulum.

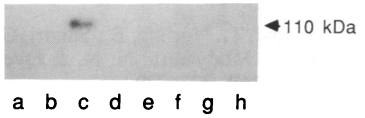

FIG. 5. Phosphorylation of a 110-kDa protein band in microsomal fractions from COS-1 cells transfected with wild-type and mutant cDNAs encoding the Ca^{2+}-ATPase. Membrane proteins were phosphorylated with [γ-^{32}P]ATP, separated on a polyacrylamide gel, and subjected to autoradiography. Nontransfected cells in the presence (lane a) and absence (lane b) of Ca^{2+}; wild-type cDNA in the presence (lane c) and absence (lane d) of Ca^{2+}; and mutants aspartic acid-351 to glutamic acid (lane e), asparagine (lane f), threonine (lane g), and alanine (lane h) in the presence of Ca^{2+}.

Expression is transient and we isolate microsomal fractions 48–72 hr after transfection. Transfection efficiency is a limiting factor in our experiments since we isolate microsomes from transfected as well as nontransfected cells. The fact that we routinely achieve such high levels of activity above a very low background activity attests to the fact that there is a high level of expression in those cells that are transfected. We have noted that our transfection results are highly reproducible from experiment to experiment, so if we use the same stocks of DNA and of COS-1 cells, the Ca^{2+} uptake rates are virtually identical from one transfection to the next.

Reported rates of oxalate-dependent Ca^{2+} uptake in the sarcoplasmic reticulum range from 1 to $\approx$4 μmol per min per mg of protein when the ATPase makes up $\approx$2/3rd of the total protein or $\approx$1.5 to 6 μmol per min per mg of ATPase protein (32). Our Ca^{2+}-uptake rates were $\approx$75 nmol per min per mg of protein when the ATPase accounts for $\approx$5% of the total protein in the microsomal preparation or $\approx$1.5 μmol per min per mg of expressed ATPase protein. This rate lies in the lower range of that reported for the sarcoplasmic reticulum.

From immunoblotting experiments with an antibody that recognizes the slow-twitch isoform of the Ca^{2+}-ATPase and from analysis of cDNA isolated from kidney libraries, we have deduced that the slow-twitch Ca^{2+}-ATPase isoform is expressed endogenously in COS-1 (monkey kidney) cells (J. Lytton, J. Fujii, K.M. and D.H.M., unpublished observations). Since this isoform does not crossreact with antibody A52, it does not appear in immunoblots. We have not attempted to inactivate this Ca^{2+}-ATPase gene in COS-1 cells, but the level of activity of this enzyme is very low, as we have demonstrated in Figs. 2 and 4, and for the purposes of our experiments, it is negligible.

The first use of our expression system was to investigate whether there is a difference in the activities of the alternatively spliced forms of the Ca^{2+}-ATPase that we have discovered in neonatal rabbit muscle (5, 6). We found that these two forms had identical Ca^{2+}-transport activities and Ca^{2+} affinities, in spite of their very different carboxyl termini. Thus, on the basis of measurement of intrinsic Ca^{2+}-transport activity, we are unable to assign any functional consequence to this developmentally regulated switch in structure. On the other hand, it is possible that the alternative carboxyl-terminal amino acid sequences provide binding sites for factors not present in COS-1 cells that do influence ATPase activity in muscle.

Mutants at Aspartic Acid-351, Lysine-352, and Lysine-515. The effectiveness of the expression system for the study of site-specific mutagenesis of the Ca^{2+}-ATPase is evident in our analysis of three residues, aspartic acid-351, lysine-352,

and lysine-515. When aspartic acid-351 was mutated to any of six other residues, the Ca^{2+}-transport function was abolished. Of particular interest was the mutation to glutamic acid. Although glutamic acid and aspartic acid differ by only one methylene group, this alteration was sufficient to prevent catalytic phosphoenzyme formation, destroying the Ca^{2+}-transport function of the protein. The mutation of aspartic acid-351 to asparagine also inactivated the enzyme.

Although F_1–F_0 type ATPases are not phosphorylated, they have a sequence similar to the phosphorylation site sequence of cation transport ATPases. In this homologous sequence, for example, in bovine mitochondria in which the sequence is -Thr-Thr-Thr*-Lys-Lys-Gly-Ser-Ile-Thr-, Thr* is replaced by aspartic acid in cation transport ATPases (33). Thus, it was of interest to mutate the aspartic acid to threonine or to serine. Again, these mutants were inactive. The final mutations were to histidine and alanine, both of which were inactive.

While it was not too surprising that mutation of aspartic acid-351 would inactivate the enzyme, it was surprising that mutation of the adjacent lysine-352 was equally devastating to the function of the protein. Mutations of lysine-352 to arginine, glutamine, or glutamic acid were all inactive, demonstrating that it is not sufficient merely to retain the positive charge on this residue. Reversal of the sequence Asp^{351}-Lys^{352} to Lys^{351}-Asp^{352} also created an inactive protein. Thus, it appears that small changes in the position of either the carboxyl group of aspartic acid-351 or the ε amino group of lysine-352 are deleterious to enzymatic activity, suggesting that a very rigid and precise alignment for these key residues is necessary for enzymatic activity. Clearly, the phosphorylation site is made up of several essential residues, which may include the whole conserved sequence surrounding this site.

In contrast to the high specificity of aspartic acid-351 and lysine-352, lysine-515 could be mutated with less devastating effects. Arginine-515 and glutamic acid-515 were partially active but mutation to glutamic acid-515, replacing a basic residue with an acidic residue, did inactivate the enzyme. Thus, activity decreased in order of the loss of positive charge, indicating that this site has some flexibility but is unable to withstand a negative charge. It is possible that this side chain interacts with a negative charge on ATP, perhaps one of the phosphoryl groups. Because these mutants have sharply diminished Ca^{2+}-transport rates, we are not yet certain whether the mutations affect ATP binding. These mutations will be of great value in further investigations aimed at understanding the reason for their reduced enzymatic activity.

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1. Ebashi, S., Endo, M. & Ohtsuki, I. (1969) *Q. Rev. Biophys.* **2,** 351–384.
2. Inesi, G. (1985) *Annu. Rev. Physiol.* **47,** 573–601.
3. MacLennan, D. H. & Reithmeier, R. A. F. (1985) in *Structure and Function of Sarcoplasmic Reticulum*, eds. Fleischer, S. & Tonomura, Y. (Academic, London), pp. 91–100.
4. MacLennan, D. H., Brandl, C. J., Korczak, B. & Green, N. M. (1985) *Nature (London)* **316,** 696–700.
5. Brandl, C. J., Green, N. M., Korczak, B. & MacLennan, D. H. (1986) *Cell* **44,** 597–607.
6. Brandl, C. J., DeLeon, S., Martin, D. R. & MacLennan, D. H. (1987) *J. Biol. Chem.* **262,** 3768–3774.
7. Allen, G. & Green, N. M. (1976) *FEBS Lett.* **63,** 188–192.
8. Pick, U. & Bassilian, S. (1981) *FEBS Lett.* **123,** 127–130.
9. Mitchinson, C., Wilderspin, A. F., Trinnaman, B. J. & Green, N. M. (1982) *FEBS Lett.* **146,** 87–92.
10. Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A. & Clark, S. C. (1985) *Science* **228,** 810–815.
11. Gluzman, Y. (1981) *Cell* **23,** 175–182.
12. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3,** 280–289.
13. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
14. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Sompayrac, L. M. & Danna, K. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7575–7578.
18. Gorman, C. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Washington, DC), Vol. 2, pp. 143–190.
19. MacLennan, D. H. & deLeon, S. (1983) *Methods Enzymol.* **96,** 570–579.
20. Resh, M. D. & Erikson, R. L. (1985) *J. Cell Biol.* **100,** 409–417.
21. Yamada, S. & Ikemoto, N. (1980) *J. Biol. Chem.* **255,** 3108–3119.
22. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (London)* **75,** 463–505.
23. Sarkadi, B., Enyedi, A., Foldes-Papp, Z. & Gardos, G. (1986) *J. Biol. Chem.* **261,** 9552–9557.
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
25. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
26. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
27. Zubrzycka-Gaarn, E., MacDonald, G., Phillips, L., Jorgensen, A. O. & MacLennan, D. H. (1984) *J. Bioenerg. Biomembr.* **16,** 441–464.
28. Allen, G., Trinnaman, B. J. & Green, N. M. (1980) *Biochem. J.* **187,** 591–616.
29. Fersht, A. R., Leatherbarrow, R. J. & Wells, T. N. C. (1986) *Nature (London)* **322,** 284–286.
30. Carrasco, N., Antes, M. L., Poonian, M. S. & Kaback, H. R. (1986) *Biochemistry* **25,** 4486–4488.
31. Hackett, N. R., Stern, L. J., Chao, B. H., Kronis, K. A. & Khorana, H. G. (1987) *J. Biol. Chem.* **262,** 9277–9284.
32. de Meis, L. (1981) *The Sarcoplasmic Reticulum* (Wiley, New York).
33. Ernster, L., Hundal, T., Norling, B., Sandri, G., Wojtczak, L., Grinkevich, V. A., Modyanov, N. N. & Ovchinnikov, Y. A. (1986) *Chem. Scr.* **26B,** 273–279.