

The Alternative Carboxyl Termini of Avian Cardiac and Brain Sarcoplasmic Reticulum/Endoplasmic Reticulum Ca^{2+} -ATPases Are on Opposite Sides of the Membrane*

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The sarcoplasmic/endoplasmic reticulum slow-twitch or cardiac Ca^{2+} -ATPase (SERCA2) is expressed as two forms (SERCA2a and SERCA2b) which vary at their extreme carboxyl termini. SERCA2a and SERCA2b are derived from alternatively spliced primary transcripts of the same gene. These two alternative carboxyl termini are highly conserved in mammals (Eggermont, J. A., Wuytack, F., De Jaegere, S., Nelles, L., and Casteels, R. (1989) *Biochem. J.* 260, 757-761; Lytton, J., and MacLennan, D. H. (1988) *J. Biol. Chem.* 263, 15024-15031) and birds (Campbell, A. M., Kessler, P. D., Sagara, Y., Inesi, G., and Fambrough, D. M. (1991) *J. Biol. Chem.* 266, 16050-16055). The topology of SERCA2a is believed to be identical to the fast-twitch Ca^{2+} -ATPase (SERCA1) with 10 membrane-spanning domains. Based on hydropathy analysis, the extended carboxyl terminus of SERCA2b is predicted to span the endoplasmic reticulum (ER) membrane an additional (*i.e.* 11th) time. We have added the human *c-myc* epitope, a 10-amino acid sequence recognized by monoclonal antibody 9E10, onto the carboxyl termini of SERCA2a and SERCA2b to test whether or not their carboxyl termini are on the same side of the ER membrane. The added epitopes do not appear to disrupt topology as judged from unaltered Ca^{2+} transport. Immunocytochemical studies demonstrate that SERCA2a and SERCA2b have their carboxyl termini on opposite sides of the ER membrane; SERCA2a's is in the cytosol and SERCA2b's is in the ER lumen.

Intracellular free Ca^{2+} concentration is maintained around $0.1 \mu\text{M}$ in part by Ca^{2+} -ATPases (SERCAs)¹ that transport two Ca^{2+} ions into the sarcoplasmic or endoplasmic reticulum per ATP molecule hydrolyzed. Three SERCA-type Ca^{2+} -ATPase genes (Brandl *et al.*, 1986; Burk *et al.*, 1989) have been identified. These encode five gene products, due to alternative splicing of SERCA1 (fast-twitch; Brandl *et al.*, 1986) and SERCA2 (slow-twitch; Lytton and MacLennan, 1988; Gunteski-Hamblin *et al.*, 1988) primary transcripts. Among all the Ca^{2+} -ATPase isoforms sequenced from birds and mammals,

there is greater than 70% amino acid identity (Burk *et al.*, 1989; Karin *et al.*, 1989; Campbell *et al.*, 1991). Since the various isoforms and the alternative carboxyl termini of SERCA2a and SERCA2b are highly conserved between birds and mammals, one can postulate that there have been selective pressures which have retained the conserved residues. However, functional comparisons have not yet revealed any differences between the multiple isoforms of the cloned SERCAs (Campbell *et al.*, 1991; Lytton *et al.*, 1991).

Immunological experiments support the topological model of SERCA1 having 10 membrane-spanning domains (Clarke *et al.*, 1990; Matthews *et al.*, 1990), originally proposed by MacLennan *et al.* (1985). When SERCA2b was first sequenced (Lytton and MacLennan, 1988; Gunteski-Hamblin *et al.*, 1988), hydropathy analysis suggested it had an additional (*i.e.* 11th) membrane-spanning domain. The same was found for the avian SERCA2b (Campbell *et al.*, 1991). Fig. 1A shows the high percentage of amino acid sequence identity between chicken, rat, human, rabbit, and pig SERCA2b. Hydropathy plots (Fig. 1B; Kyte and Doolittle, 1982) of avian SERCA2a and SERCA2b, beginning with the last two common membrane-spanning domains, illustrate the point that SERCA2b might span the ER membrane once more than SERCA2a. The resulting topology is depicted in Fig. 1C.

To determine whether or not the carboxyl termini of SERCA2a and SERCA2b are on opposite sides of the ER membrane, we combined immunocytochemical techniques and a procedure to selectively permeabilize the plasma membrane with the bacterial toxin streptolysin-O (SLO).² Initially, we employed polyclonal antisera specific for SERCA2a or SERCA2b termini (generously provided by F. Wuytack and R. Casteels (Katholieke Universiteit, Leuven, Belgium); Eggermont *et al.*, 1990; Wuytack *et al.*, 1989) but were unable to get strong signals. Therefore, we decided to add onto the carboxyl termini of SERCA2a and SERCA2b a 10-amino acid sequence (EQKLISEEDL) derived from the human oncogene product *c-myc*, recognized by mAb 9E10 (Evan *et al.*, 1985). These epitope-tagged constructs, SERCA2a-myc and SERCA2b-myc, were used to determine the location of the carboxyl termini.

EXPERIMENTAL PROCEDURES

***c-myc* Tag cDNA Constructs**—The endogenous stop codons in the cDNAs encoding SERCA2a and SERCA2b were deleted and replaced by the *c-myc* tag encoding nucleotides. A polymerase chain reaction

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¹ The nomenclature used here was adopted from Burk *et al.* (1989).

² The abbreviations used are: SLO, streptolysin-O; COS-1, monkey kidney tumor cell line transformed with SV40 large-T antigen; EGTA, [ethylenbis(oxyethylenenitrilo)]tetraacetic acid; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum.

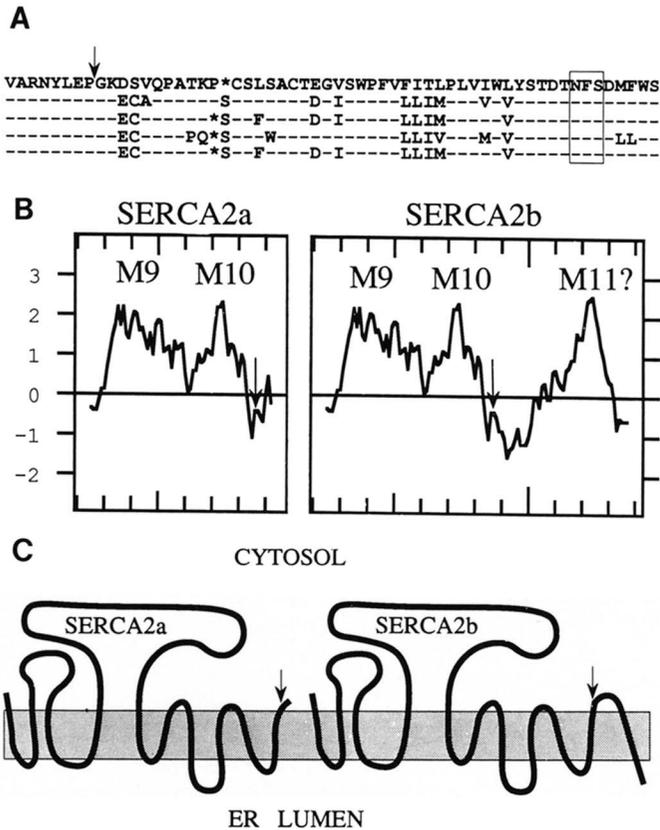


FIG. 1. *A*, comparison of partial SERCA2b sequences using the one-letter amino acid code, from *top to bottom*: chicken, rat, human, rabbit, and pig. The *dashes* indicate identical residues; the *asterisks* are gaps to maximize alignment; the *arrow* indicates the alternative splice site; and the *box* encloses the conserved asparagine-linked glycosylation consensus sequence NFS. *B*, hydrophathy plots, using the Kyte and Doolittle (1982) program with a window set for 12 amino acids of chicken SERCA2a and SERCA2b beginning with amino acid 920. Positive numbers are hydrophobic, and negative numbers are hydrophilic; *hash marks* on the *x* axis demark units of 10 amino acids; M9, M10, and M11 indicate the predicted membrane-spanning domains; the *arrows* indicate the alternative splice sites. *C*, cartoons depicting SERCA2a and SERCA2b topologies if the two forms have 10 and 11 membrane-spanning domains, respectively. The *shaded box* represents the ER membrane, and the *arrows* mark the alternative splice site and thus sequence divergence.

(PCR)³-based technique was used to add on the *c-myc* encoding nucleotides, and conditions were as recommended by the Amplitaq DNA polymerase instructions (Perkin-Elmer Cetus). Thirty temperature cycles of 95 °C, 50 °C (for 1 min), and 72 °C (for 20 s) were performed to produce the *c-myc*-tagged SERCA2a and SERCA2b carboxyl termini encoding cDNA. The *c-myc* tags encoding PCR primers were as follows: SERCA2a-myc, 5' TGTGTGGGTACCTA GAGTCTTCCTCAGAGATCAGCTTCTGCTC 3'; SERCA2b-myc, 5' TGTGTGGGTACCTAGAGGTCTTCCTCAGAGATCAGC TTCTGCTCAGACCAGAACATATCAAAG 3'. These primers contain a *KpnI* site for cloning, a stop codon, the *c-myc* epitope (EQKLISEEDL) encoding nucleotides, and 20 bases of the appropriate avian sequence encoding the carboxyl terminus of either SERCA2a or SERCA2b. The other primer was derived from the cDNA sequence on the 5' side of the unique internal *SalI* site (Campbell *et al.*, 1991). The PCR products were digested with *KpnI* and *SalI*, purified and cloned into pBluescript (Stratagene), and sequenced to ensure that there were no mutations. These were then cloned into the internal *SalI* site of SERCA2. The resulting SERCA2a-myc and SERCA2b-myc cDNAs were cloned into the *KpnI* site of the expression vector described below.

Expression in Tissue Culture—cDNAs of SERCA2a, SERCA2b,

³ PCR, polymerase chain reaction, is patented by Perkin-Elmer Cetus.

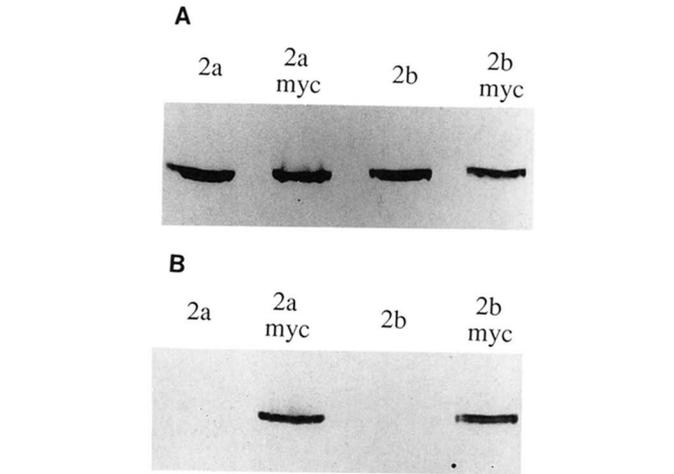


FIG. 2. Immunoblots of microsomes made from cells transfected with cDNA encoding SERCA2a, SERCA2a-myc, SERCA2b, or SERCA2b-myc as indicated. The proteins were detected with avian SERCA2-specific mAb 3H2 (*panel A*) or human *c-myc*-specific mAb 9E10 (*panel B*). Equal amounts of protein (15 µg/lane) were loaded in each lane.

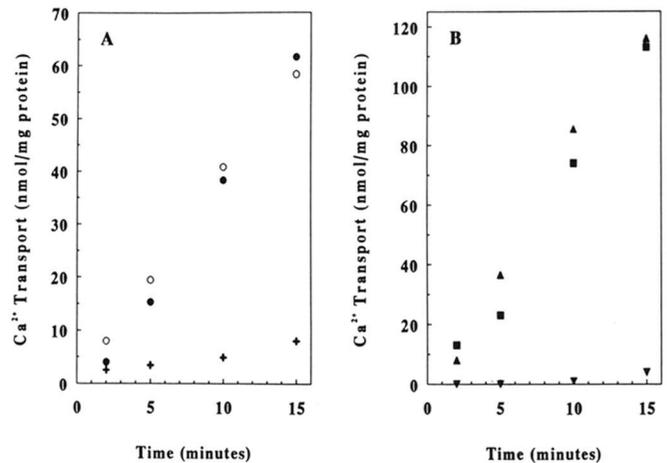


FIG. 3. Calcium uptake comparisons between tagged and wild-type Ca²⁺-ATPases. *Panel A* compares wild-type SERCA2a (●) with SERCA2a-myc (○) and SERCA2a-myc preincubated with thapsigargin (+). *Panel B* compares wild-type SERCA2b (■) with SERCA2b-myc (▲) and SERCA2b-myc preincubated with thapsigargin (▼). The *myc*-tagged data points (▲ and ○) are the average of two independent experiments. Wild-type and thapsigargin data points are each results of single experiments. ⁴⁵Ca²⁺ uptake by microsomes made from mock transfected cells was always less than 10% of ⁴⁵Ca²⁺ uptake by microsomes made from transfected cells and has been subtracted from all the time points. The microsomes used within a *panel* were prepared on the same day from cells transfected on the same day.

SERCA2a-myc, and SERCA2b-myc were cloned into the *KpnI* site of the expression vector pcDL-SRa296 (Takebe *et al.*, 1988). COS-1 cells were transfected using DEAE dextran and 10% dimethyl sulfoxide as in Campbell *et al.* (1991).

Microsome Preparation—As in Campbell *et al.* (1991), transfected COS-1 cells were harvested and then homogenized with a Dounce homogenizer. The suspension was centrifuged at 10,000 × *g* for 20 min, and the supernatant was centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended with 50 mM MOPS (pH 7.0), 10% sucrose and frozen in liquid nitrogen for later use. Total microsomal protein concentrations were determined with the Bio-Rad protein assay reagent.

Immunoblot Analysis—15 µg of microsomal protein from cells transfected with SERCA2a, SERCA2a-myc, SERCA2b, and SERCA2b-myc were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel (Laemmli, 1970), transferred to nitrocellulose, and incubated in 10 mM Tris (pH

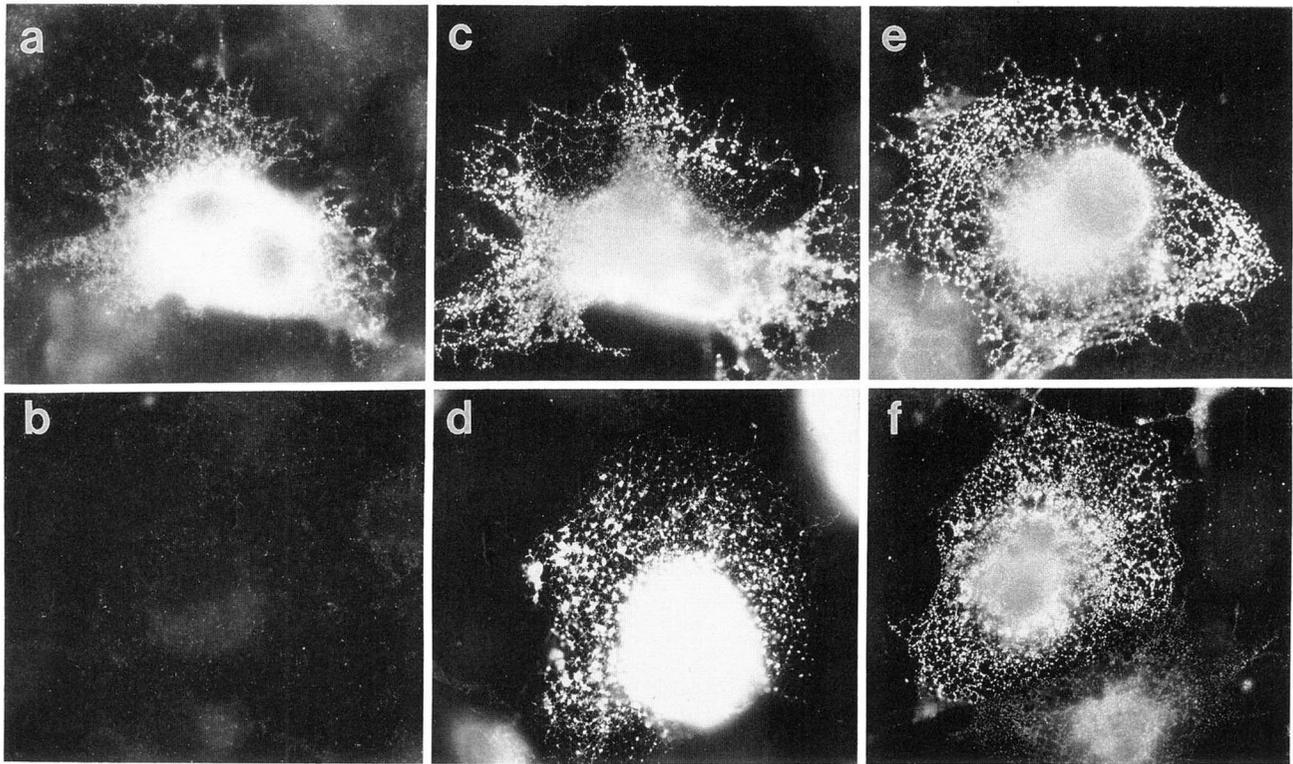


FIG. 4. Immunofluorescence photomicrographs of COS-1 cells transfected with cDNA encoding SERCA2a-myc (panels a, c, and e) or SERCA2b-myc (panels b, d, and f). Panels a and b were permeabilized with SLO alone and stained with mAb 9E10. Panels c and d were stained with mAb 9E10 after permeabilization with SLO and saponin. Panels e and f were permeabilized with SLO alone and stained with mAb 3H2. The exposure time for panel B was identical to that of panel A. All photomicrographs were taken with the $\times 63$ objective lens and printed to the same magnification.

7.4), 150 mM NaCl, 5% dry milk, 0.02% Tween 20 (Fisher), and 0.01% Antifoam A (Sigma), and either 5 $\mu\text{g}/\text{ml}$ mAb 3H2, specific to avian SERCA2 (CaS-3H2; Kaprielian and Fambrough, 1987) or 10 $\mu\text{g}/\text{ml}$ mAb 9E10, specific for the human *c-myc* epitope (Evan *et al.*, 1985). The bound antibodies were detected as described in the enhanced chemiluminescence protocol (Amersham Corp.). Expression levels of all forms of SERCA2 were measured by ¹²⁵I-labeled mAb 3H2 binding as in Campbell *et al.* (1991). Wild-type SERCA2a and SERCA2a-myc expression levels were comparable, as were wild-type SERCA2b and SERCA2b-myc. However, SERCA2a and SERCA2a-myc expression levels were about 60% of SERCA2b and SERCA2b-myc expression levels. This is consistent with previous results (Campbell *et al.*, 1991).

Calcium Uptake Assay—5.0 ml of the reaction mixture (20 mM MOPS, pH 7.0, 60 mM KCl, 5 mM MgCl₂, 5 mM sodium oxalate, 0.2 mM EGTA, 0.2 mM CaCl₂ (with 0.4 $\mu\text{Ci}/\text{ml}$ ⁴⁵Ca²⁺) and 10 $\mu\text{g}/\text{ml}$ microsomal protein) was equilibrated at 25 °C for 5 min before 2.5 mM ATP was added at time zero. 1.0-ml aliquots were removed at timed intervals, filtered (0.45 μm pore size; Millipore), and washed with 12 ml of 10 mM MOPS (pH 7.0), 2 mM LaCl₃. A liquid scintillation counter was used to determine amounts of ⁴⁵Ca²⁺ on the filter. For thapsigargin (Bethesda Research Laboratories) inhibition, 20 nM thapsigargin was present during the 5-min incubation period. The difference between Fig. 3A and Fig. 3B in the nanomoles of Ca²⁺ transported per mg of total microsomal protein is probably a result of variations in transfection efficiency and/or protein accumulation.

Immunofluorescence—Cells were plated on multiple coverslips placed in the same 100-mm tissue culture dish to ensure the uniformity of transfections. Therefore, cells depicted in Fig. 4, a, c, and e (SERCA2a-myc) were transfected simultaneously, as were the cells in Fig. 4, b, d, and f (SERCA2b-myc). Transfected cells were permeabilized with SLO (Burroughs Wellcome, Research Triangle Park, NC) by a method modified from Gravotta *et al.* (1990). In order to preserve the reticular morphology of the ER, the SLO was used at 1.6 units/ml in a sodium-free buffer (25 mM HEPES, pH 7.4, 2.5 mM magnesium acetate, 25 mM KCl, 250 mM sucrose). After the cells were permeabilized, they were fixed in buffered 1% formaldehyde for 10 min at room temperature and blocked with buffered 1% bovine

serum albumin and 5 mg/ml L-lysine. Then the cells were incubated with mAb 9E10 or mAb 3H2 in buffered 1% BSA either with or without 0.25% saponin as indicated, followed by a fluorescein-conjugated goat anti-mouse secondary antibody, and photographed on an epifluorescence microscope. Cells labeled for immunofluorescence microscopy are referred to as “stained.”

RESULTS AND DISCUSSION

To verify the cDNA constructs and Ca²⁺-ATPase identities, microsomal proteins derived from COS-1 cells transfected with cDNAs encoding SERCA2a, SERCA2a-myc, SERCA2b, or SERCA2b-myc were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with mAb 3H2 or mAb 9E10 (Fig. 2). mAb 3H2 identified the wild-type chicken SERCA2a and SERCA2b, as well as their *myc*-tagged derivatives (Fig. 2A). mAb 9E10 recognized the human *c-myc* epitope that had been added to the carboxyl termini of SERCA2a and SERCA2b (Fig. 2B). Fig. 2B demonstrates that the correct microsomal preparations have expressed the *myc*-tagged ATPases and that wild-type SERCA2s do not react with mAb 9E10. The slight difference in molecular weights between SERCA2a-myc and SERCA2b-myc can be detected as a difference in electrophoretic mobility (Fig. 2B, lanes 2 and 4).

Epitope tags have been added to a variety of proteins in order to answer questions about topology and function. The human *c-myc* epitope has been used frequently for this purpose (Peculis and Gall, 1992; Ellison and Hochstrasser, 1991; reviewed in Kolodziej and Young (1991)). The caveat with epitope tagging of proteins is that the added epitope might disrupt native topology. If the topology were aberrant, then the misfolded protein would probably accumulate in the ER.

For a protein which normally leaves the ER, subcellular localization of the tagged protein in the ER would reveal the misfolding. However, SERCA2 is an ER resident protein, so another assay was necessary to examine whether or not the added epitope disrupted the topology of SERCA2a-myc and SERCA2b-myc.

The best available assay to determine native conformation is a functional one. We measured the relative ability of the *myc*-tagged Ca²⁺-ATPases and wild-type Ca²⁺-ATPases to transport Ca²⁺. Microsomes were made from COS-1 cells after transfection with SERCA2a, SERCA2a-myc, SERCA2b, or SERCA2b-myc encoding cDNAs, and ATP-dependent uptake of ⁴⁵Ca²⁺ was measured (see "Experimental Procedures"). The results (Fig. 3, A and B) show that Ca²⁺-ATPases with or without the *c-myc* epitope are equally competent to transport Ca²⁺. As a further test of native topology, thapsigargin, a potent inhibitor of both SERCA2a and SERCA2b (Campbell *et al.*, 1991), was added to the reaction mixture. Both wild-type and *myc*-tagged Ca²⁺-ATPases were inhibited by thapsigargin.

Having shown SERCA2a-myc and SERCA2b-myc to be functionally unaltered, we addressed the question of topology using immunocytochemical techniques. The initial objective was to make the plasma membrane permeable to antibodies without perturbing the membrane of the ER. To achieve this, we utilized the bacterial cytolytic protein SLO. SLO is a 69-kDa protein which binds to cholesterol in a concentration-dependent manner. When mammalian cells were treated so that their cholesterol content fell by 50%, the cells became resistant to high concentrations of SLO (Duncan and Buckingham, 1980). Since the cholesterol concentration of the plasma membrane is 3–6 times higher than that of the ER (Keenan and Morr e, 1970; Colbeau *et al.*, 1971), it is not surprising that SLO was unable to permeabilize the ER sufficiently to allow antibodies access to the ER lumen (see below). To further ensure no internal membranes were permeabilized by SLO, a two-step procedure was employed (see "Experimental Procedures"). With high concentrations of SLO, lesions of about 13 nm can be produced in the plasma membrane (Buckingham and Duncan, 1983) through which antibodies can pass. Using this approach, the topology of SERCA2a-myc and SERCA2b-myc could be immunologically determined in whole cells with their ER membranes left intact.

Cells transfected with SERCA2a-myc or SERCA2b-myc encoding cDNAs were fixed, permeabilized with SLO or SLO plus saponin, which will permeabilize the ER as well as the plasma membrane. The cells were stained with mAb 9E10 (anti-*myc*) or mAb 3H2 (anti-SERCA2) as outlined under "Experimental Procedures." Using SLO and mAb 9E10 without saponin, cells expressing SERCA2a-myc showed an intense staining around the nucleus (out of the focal plane) and a reticular/punctate staining pattern throughout the cell (Fig. 4a) that is characteristic of the ER (Louvard *et al.*, 1982). However, there was no specific staining in similarly treated cells transfected with SERCA2b-myc cDNA (Fig. 4b). To verify that mAb 9E10 was able to label comparable levels of SERCA2b-myc and SERCA2a-myc, simultaneously transfected cells were permeabilized with SLO and saponin to reveal all *c-myc* epitopes (Fig. 4, c and d). There are about 10⁵ cells on each coverslip, and our transfection efficiency is about 10%. In all cases where antibody labeling was positive, there were about 10,000 positively stained cells. However, on coverslips with SERCA2b-myc-transfected cells treated with SLO and mAb 9E10 without saponin (Fig. 4b), there were zero positively stained cells. This experiment has been repeated

three times. Therefore the carboxyl terminus of SERCA2a-myc is cytosolic while that of SERCA2b-myc is luminal.

Additional controls were performed to confirm that the ER was not permeabilized by SLO. The *c-myc* epitope was placed on the luminal domain of chicken Na/K-ATPase β_2 subunit.⁴ There was no intracellular mAb 9E10 staining in β_2 -myc transfected cells permeabilized with SLO alone, but intense ER staining was present in transfected cells permeabilized with saponin (data not shown). This verified that mAb 9E10 has access to the ER lumen when saponin is used for permeabilization but not SLO. As another test, we employed a mAb to an epitope of rat dipeptidyl peptidase IV (Bartles *et al.*, 1985) with a luminal orientation. When COS-1 cells were transfected with dipeptidyl peptidase IV cDNA (Ogata *et al.*, 1989) and immunofluorescently stained, there was no intracellular labeling in SLO-permeabilized cells but intense ER labeling of saponin-permeabilized cells was present.

We chose saponin to permeabilize the internal membranes because SLO and saponin (see Birk and Peri (1980)) are both cholesterol-specific. It is unlikely, therefore, that saponin would alter protein conformation and thus artifactually expose SERCA2a-myc or SERCA2b-myc epitopes. To confirm this, Ca²⁺-dependent ATPase measurements (Lin and Morales, 1977) were conducted on rabbit light SR in the presence or absence of the same concentrations of saponin as used in Fig. 4, c and d. The ATPase activity of the Ca²⁺-ATPase is retained in the presence of saponin. Similar results were obtained using C12E8 (data not shown; de Foresta *et al.*, 1989). Therefore, it is very unlikely that the *myc* epitope on SERCA2b-myc was in the cytosol but inaccessible to mAb 9E10 (Fig. 4b) and became more accessible in the presence of saponin (Fig. 4d). Fig. 4, e and f, shows cells treated with SLO in the absence of any detergent and stained with mAb 3H2 to demonstrate cytosolic localization of the mAb 3H2 epitope, which is between amino acids 200 and 260 of SERCA2.

Why evolution has resulted in two similar forms of SERCA2 is not yet understood. The different topologies of SERCA2a and SERCA2b suggest a model to differentially regulate the two Ca²⁺-ATPases. Perhaps one of the many Ca²⁺ binding proteins of the ER acts as an accessory factor and interacts with the luminal carboxyl terminus of SERCA2b. This would allow luminal Ca²⁺ concentration-dependent regulation of SERCA2b. Furthermore, this mechanism would enable an individual cell, which expresses both SERCA2a and SERCA2b, to regulate the two Ca²⁺-ATPases differentially. An example of such cells are cerebellar Purkinje cells which Plessers *et al.* (1991) have shown predominantly express SERCA2b but also express SERCA2a.

We have demonstrated that each form of SERCA2 has its carboxyl terminus in a different subcellular environment. Functional comparisons of SERCAs conducted in tissue-cultured COS cells (Campbell *et al.*, 1991; Lytton *et al.*, 1991) where the postulated regulatory factor(s) may not exist have revealed no differences among the various forms. It has been demonstrated that different tissues preferentially express either SERCA2a or SERCA2b (Lytton and MacLennan, 1988; Plessers *et al.*, 1991; Campbell *et al.*, 1991) so future studies might focus on tissues and cell types which may contain the hypothesized regulatory factors (*e.g.* brain). If regulatory factors were discovered, then we would finally begin to understand the selective pressures which resulted in distinct Ca²⁺ stores and Ca²⁺-ATPases to be highly conserved in birds and mammals. Nevertheless, we now have the first clue to functional and evolutionary differences of the nearly identical

⁴ V. Lemas, K. Takeyasu, and D. Fambrough, manuscript in preparation.

Ca²⁺-ATPases: SERCA2a and SERCA2b.

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