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

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The sarcoplasmic/endoplasmic reticulum slow-twitch or cardiac Ca\textsuperscript{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 Jaeger, S., Nelles, L., and Castels, 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} concentration is maintained around 0.1 μM in part by Ca\textsuperscript{2+}-ATPases (SERCAs) that transport two Ca\textsuperscript{2+} ions into the sarcoplasmic or endoplasmic reticulum per ATP molecule hydrolyzed. Three SERCA-type Ca\textsuperscript{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 the Ca\textsuperscript{2+}-ATPase isoforms sequenced from birds and mammals, there is greater than 70% amino acid identity (Burk et al., 1989; Karin et al., 1988; 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). Initially, we employed polyclonal antisera specific for SERCA2a or SERCA2b termini (generously provided by F. Wytack and R. Castels (Katholieke Universiteit, Leuven, Belgium); Eggermont et al., 1990; Wytack 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., 1986). 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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‡ The abbreviations used are: SLO, streptolysin-O; COS-1, monkey kidney tumor cell line transformed with SV40 large-T antigen; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum.

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 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, hydropathy 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 carboxy termini encoding cDNA. The c-myc tags encoding PCR primers were as follows: SERCA2a-myc, 5‘-TGTGTGGGTACCTAGAGGTCTTCCTCAGAGATCAGC

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 SH2 (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. Ca²⁺ uptake by microsomes made from mock transfected cells was always less than 10% of Ca²⁺ 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.

Expression in Tissue Culture—cDNAs of SERCA2a, SERCA2b, 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

³ PCR, polymerase chain reaction, is patented by Perkin-Elmer Cetus.
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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 ×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 μg/ml mAb 3H2, specific to avian SERCA2 (Cas-3H2; Kaprielian and Fambrough, 1987) or 10 μg/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 0.25%-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 MgCl\textsubscript{2}, 5 mM sodium oxalate, 0.2 mM EGTA, 0.2 mM CaCl\textsubscript{2} (with 0.4 μCi/ml \textsuperscript{45}Ca\textsuperscript{2+}) and 0.2 mM CaCl\textsubscript{2}) 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 LaCl\textsubscript{3}. A liquid scintillation counter was used to determine amounts of \textsuperscript{45}Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} 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 e (SERCA2b-myc). Transfected cells were permeabilized with SLO (Burroughs Welcome, 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 KC\textsubscript{1}, 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 Ca\textsuperscript{2+}-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. 2B, lanes 2 and 4). mAb 9E10 recognized the human c-myc epitope that had been added to the carboxyl termini of SERCA2a and SERCA2b, and probes 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). 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 Ca\(^{2+}\)-ATPases and wild-type Ca\(^{2+}\)-ATPases to transport Ca\(^{2+}\). Microsomes were made from COS-1 cells after transfection with SERCA2a, SERCA2a-myc, SERCA2b, or SERCA2b-myc encoding cDNAs, and ATP-dependent uptake of \(^{45}\)Ca\(^{2+}\) was measured (see “Experimental Procedures”). The results (Fig. 3, A and B) show that Ca\(^{2+}\)-ATPases with or without the c-myc epitope are equally competent to transport Ca\(^{2+}\). 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 Ca\(^{2+}\)-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 Morré, 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-SERCA2a) 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 10\(^6\) 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 \(\beta_2\) subunit. There was no intracellular mAb 9E10 staining in \(\beta_2\)-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 artificially expose SERCA2a-myc or SERCA2b-myc epitopes. To confirm this, Ca\(^{2+}\)-dependent ATPase measurements (Lin and Morris, 1977) were conducted on rabbit light SR in the presence of the same concentrations of saponin as used in Fig. 4, c and d. The ATPase activity of the Ca\(^{2+}\)-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 SERCA2a.

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 Ca\(^{2+}\)-ATPases. Perhaps one of these Ca\(^{2+}\)-binding proteins of the ER acts as an accessory factor and interacts with the luminal carboxyl terminus of SERCA2b. This would allow luminal Ca\(^{2+}\) concentration-dependent regulation of SERCA2b. Furthermore, this mechanism would allow an individual cell, which expresses both SERCA2a and SERCA2b, to regulate the two Ca\(^{2+}\)-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 Ca\(^{2+}\) stores and Ca\(^{2+}\)-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 SERCA2a and SERCA2b.
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Ca\textsuperscript{2+}-ATPases: SERCA2a and SERCA2b.

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