Structure-Function Analysis of the SERCA2b C-terminal Tail

by

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ABSTRACT

The sarcoplasmic and endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) 2 gene encodes two protein isoforms: SERCA2a and SERCA2b. Structurally, they differ at their C-termini. Compared with SERCA2a, SERCA2b has a higher apparent affinity for calcium and a two-fold lower turnover rate. The role of the C-terminal region in determining the SERCA2b phenotype was tested by mutating Asn1035 of human SERCA2b to Ala, creating progressive deletions of increasing length from the C-terminus, and creating a mutant with an extra Leu introduced in the middle of the 11th transmembrane segment. Our data demonstrated that the Asn1035Ala mutation, which destroys the putative glycosylation site, did not influence the SERCA2b phenotype. All the deletion mutants and the insertion construct showed activities significantly different from SERCA2b and similar to SERCA2a. These experiments suggest that the last 3 amino acids in the luminal tail, as well as the orientation of the tail, are important in determining SERCA2b activity.
ACKNOWLEDGEMENTS

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I also would like to express my appreciation to my parents and sisters, as well as to all my friends who support and care for me all the time.
Dedicated to

my parents
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<tr>
<td>AM</td>
<td>Amplitude modulation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaM kinase II</td>
<td>Calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>E-C coupling</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-amino-ethyl)-N,N,N′,N′-tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FM</td>
<td>Frequency modulation</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>Human embryonic kidney cell line</td>
</tr>
<tr>
<td>ICrAC</td>
<td>Calcium release-activated calcium current</td>
</tr>
<tr>
<td>InsP3</td>
<td>Inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>InsP3R</td>
<td>Inositol 1, 4, 5-trisphosphate receptor</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic and endoplasmic reticulum Calcium-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TMS</td>
<td>Transmembrane segment</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION

A. The importance of Ca\textsuperscript{2+} and its regulation

It is well known that intracellular ionized calcium ([Ca\textsuperscript{2+} \textsubscript{i}]) is the most common signal transduction element in cells ranging from prokaryotic to eukaryotic. It is involved in every aspect of cell life: it triggers cell fertilization, controls cell development and cell differentiation, and then mediates subsequent cellular activities including cell growth, gene transcription, cell transformation, secretion, contraction and relaxation of muscle cells, neuromodulation and synaptic plasticity, as well as programmed cell death (Berridge, 1993 and Clapham, 1995). Almost everything we do is controlled by Ca\textsuperscript{2+} - how we make a movement, how our hearts beat, and how our brains process thought and memorize information (Berridge, et al., 1998).

A.1. The generation of Ca\textsuperscript{2+} signals

Cells use two sources of Ca\textsuperscript{2+} to generate signals: Ca\textsuperscript{2+} released from intracellular stores such as the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER), and Ca\textsuperscript{2+} that enters across the plasma membrane, through ion channels located in the two membrane systems. Both the Ca\textsuperscript{2+} release and entry mechanisms can give rise to small bursts of Ca\textsuperscript{2+} (elemental events) in the cytosol for signal transduction. Cell Ca\textsuperscript{2+} signals result from the summation in space and time of many of these events.

It is believed that the ER in non-muscle cells and the SR in muscle cells store the majority of the mobile intracellular Ca\textsuperscript{2+} pool (Ghosh, et al., 1989; Renard-Rooney, et al., 1993; Short, et al., 1993). Most of the elementary events reported so far are associated with the release of Ca\textsuperscript{2+} from these intracellular stores, which is controlled by two types of
channels, known as the inositol 1,4,5-trisphosphate receptor (InsP$_3$R) and the ryanodine receptor (RyR). Multiple isoforms of these receptors have been identified, which are expressed in a tissue-specific manner (De Smedt, et al., 1994). Structurally, InsP$_3$R and RyR both exist as tetramers, with the C-terminal region of each subunit co-operating to form the Ca$^{2+}$ channel, and the large N-terminal domains folding to form a complex structure in the cytoplasm (Furuichi, et al., 1989; Wagenknecht and Radermacher, 1997).

In electrically nonexcitable cells, such as blood leukocytes, Ca$^{2+}$ release from the ER is predominantly induced by the inositol (1,4,5)-trisphosphate (InsP$_3$)-mediated pathway (Berridge and Irvine, 1989). InsP$_3$, produced under the influence of the G protein-coupled receptor class of seven transmembrane-spanning receptors or receptor tyrosine kinases, acts as an intracellular second messenger by binding to the InsP$_3$ receptor that spans the ER membrane, resulting in the release of Ca$^{2+}$ from the ER. This InsP$_3$-mediated pathway can increase [Ca$^{2+}$]$_i$ from $-100$ nm to $-1$ μm.

In excitable cells such as neurons or muscle cells, depolarization of the cell membrane initiates conformational changes in the voltage-gated L-type Ca$^{2+}$ channels (dihydropyridine receptors, DHPRs) located in the plasma membrane. The process of coupling the chemical and electrical signals at the cell surface to the intracellular release of Ca$^{2+}$ is called excitation-contraction coupling (E-C coupling) in the case of muscle, and excitation-secretion coupling at the synapse between neurons (Fabiato, 1985; Catterall, 1991). In cardiac muscle, the entry of extracellular Ca$^{2+}$ through the DHPR triggers Ca$^{2+}$ release from the SR in a process referred to as Ca$^{2+}$-induced Ca$^{2+}$ release (Fabiato, 1983). In contrast, Ca$^{2+}$ entering skeletal muscle cells from the exterior through voltage-gated Ca$^{2+}$ channels is not required for initiating E-C coupling. Instead, the depolarization of the transverse tubule membrane triggers the Ca$^{2+}$ release through RyRs in the SR via a
physical interaction, called mechanical coupling. This is possible because the DHPRs and RyRs are closely co-localized (Rios and Pizarro, 1991).

There are fewer examples of the elemental events associated with Ca\textsuperscript{2+} entry. Electrically excitable cells have voltage-gated Ca\textsuperscript{2+} channels that enable these cells to increase cytosolic Ca\textsuperscript{2+} levels (Clapham, 1995). In contrast, Ca\textsuperscript{2+} may enter nonexcitable cells by crossing the plasma membrane through voltage-independent Ca\textsuperscript{2+}-selective channels triggered either by second-messenger molecules, or indirectly by the depletion of intracellular Ca\textsuperscript{2+} stores by InsP\textsubscript{3} (Clapham, 1995). This latter mechanism was originally proposed by Putney and termed capacitative Ca\textsuperscript{2+} influx (Putney, 1986). It is the primary mechanism by which cell surface receptors activate Ca\textsuperscript{2+} influx across the plasma membrane in nonexcitable cells. Numerous groups have identified multiple types of ion channels underlying capacitative Ca\textsuperscript{2+} influx (Parekh and Penner, 1997) using patch clamp techniques. These channels are referred to as store-operated Ca\textsuperscript{2+} channels on the basis of their Ca\textsuperscript{2+} permeability and activation by agents that empty Ca\textsuperscript{2+} stores. Of the store-operated Ca\textsuperscript{2+} currents, the best characterized is Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} current (I\textsubscript{CRAC}), which was first found in mast and T cells (Parekh and Penner, 1997; Hoth and Penner, 1992). I\textsubscript{CRAC} appears to be so far the most effective and widespread Ca\textsuperscript{2+} influx pathway in nonexcitable cells. Multiple types of CRAC channels have been identified in various of cells on the basis of differing Ca\textsuperscript{2+} permeability and activation by pharmacological reagents that empty Ca\textsuperscript{2+} stores (Fasolato, Innocenti and Pozzan, 1994). CRAC channels are distinguished from other types of depletion-activated Ca\textsuperscript{2+} channels by their remarkable selectivity for Ca\textsuperscript{2+} over monovalent and other divalent cations (Lewis and Cahalan, 1989; Hoth and Penner, 1992; McDonald et al., 1993; Zweifach and Lewis, 1993; Hoth and Penner, 1993; Premack et al., 1994). In the absence of Ca\textsuperscript{2+}, CRAC
channels conduct Na+, and the single-channel conductance of the CRAC channel for Na+ was reported to be 36 to 40 picoSiemens in Jurkat T lymphocytes (Kerschbaum and Cahalan, 1999).

The *trp* channel cloned from *Drosophila* photoreceptors is considered to be the first recombinant channel that can be activated by store depletion (Montell and Rubin, 1989; Petersen, et al., 1995). More recently, Zhu, et al (1996) reported the existence of six *trp*-related genes in the mouse genome. They also found that the expression of two human *trp* homologs, Htrp1 and Htrp3, in COS cells enhanced capacitative Ca\(^{2+}\) entry. According to hydropathy analysis, Htrp3 was proposed to be a six-spanning transmembrane protein. The mechanisms linking the Ca\(^{2+}\) content of the InsP\(_3\)-sensitive intracellular store to CRAC channel activity in the plasma membrane are still not known. A recent report, however, suggested a tight functional interaction between store-operated channels and InsP\(_3\) receptors (Kiselyov, et al., 1998).

The elementary signals described above can either activate highly localized cellular processes in the immediate vicinity of the channels or coordinate to generate a global [Ca\(^{2+}\)]\(_i\) increase. For example, for Ca\(^{2+}\) entry units, voltage-gated Ca\(^{2+}\) channels can be recruited in a stochastic manner following membrane depolarization although they do not communicate readily with one another. Thus, the membrane localization of the channels plus the extent of membrane depolarization determines the spatial properties of the Ca\(^{2+}\) signal. On the other hand, the coordination of elementary Ca\(^{2+}\) release from intracellular stores to produce global responses is achieved through individual release sites communicating with each other using Ca\(^{2+}\) as a messenger, i.e., Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Yao, et al., 1995). If cells are connected, such intracellular waves can travel from cell to cell and become intercellular waves to coordinate cellular response within a tissue.
(Berridge, 1993). Table 1 is a summary of the spatial aspects of Ca^{2+} signalling (Berridge, et al., 1998).

A.2. The spatiotemporal regulation of Ca^{2+} signalling

Ca^{2+} signals are both very flexible and precisely regulated, and control many diverse cellular processes. Cells respond uniquely and differentially to combinations of Ca^{2+} signals that differ in their precise parameters, such as space, time and amplitude. This is the so-called phenomenon of amplitude and frequency modulation (AM and FM) (Berridge, 1997). An example of AM regulation is the observation that low concentrations of Ca^{2+} activate the nuclear factor of activated T cells and the extracellular receptor kinase pathway, while a much larger elevation stimulates a different set of transcriptional regulators (such as NF-kB and c-Jun kinase) (Dolmetsch, et al., 1997; Genot, et al., 1996). AM signalling is generally considered to be less reliable than FM signalling owing to the difficulties of detecting small Ca^{2+} changes above the background level (Berridge, 1997). For the FM mode, the Ca^{2+} signal appears as regular oscillations whose frequency changes with the concentration of the incoming signal. FM signalling is used to control the rate of specific cellular processes such as fluid secretion by salivary glands (Rapp, 1981), glycogen metabolism by liver cells (Woods, 1986), as well as neuronal differentiation (Gu and Spitzer, 1995). Recent publications even suggested that Ca^{2+} oscillations can increase the efficiency and specificity of gene expression (Dolmetsch, et al., 1998; Li, et al., 1998).

To use FM signalling, cells have developed decoders that respond to the frequency and longevity of the Ca^{2+} signals. In 1998, De Koninck et al. used rapid superfusion of Ca^{2+} over Ca^{2+-} and calmodulin-dependent protein kinase II (CaM kinase II) to show that CaM kinase II could decode the frequency of Ca^{2+} spikes into distinct amounts of kinase activity. The frequency response of CaM kinase II was modulated by the amplitude and
Table 1. Spatial aspects of Ca\textsuperscript{2+} signalling*

<table>
<thead>
<tr>
<th>Elementary events</th>
<th>Global Ca\textsuperscript{2+} waves (intracellular)</th>
<th>Global Ca\textsuperscript{2+} waves (intercellular)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth-cone migration</td>
<td>Fertilization</td>
<td>Wound healing</td>
</tr>
<tr>
<td>Membrane excitability</td>
<td>Gene transcription</td>
<td>Ciliary beating</td>
</tr>
<tr>
<td>Mitochondrial metabolism</td>
<td>Cell proliferation</td>
<td>Glial cell function</td>
</tr>
<tr>
<td>Vesicle secretion</td>
<td>Liver metabolism</td>
<td>Bile flow</td>
</tr>
<tr>
<td>Smooth muscle relaxation</td>
<td>Muscle contraction</td>
<td>Insulin secretion</td>
</tr>
<tr>
<td>Mitosis</td>
<td></td>
<td>Smooth muscle-induced nitric oxide synthesis in endothelium</td>
</tr>
<tr>
<td>Synaptic plasticity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from Berridge, et al., 1998.
duration of individual Ca\textsuperscript{2+} spikes as well as the subunit composition and previous state of activation of the kinase (De Koninck and Schulman, 1998). Therefore, the ability of cells to transmit information is greatly enhanced by using both frequency and amplitude modulation.

A.3. The importance of Ca\textsuperscript{2+} pumps, Ca\textsuperscript{2+} antiporters and Ca\textsuperscript{2+}-binding proteins

Ca\textsuperscript{2+} is essential for cell survival, but prolonged high [Ca\textsuperscript{2+}]\textsubscript{i} levels result in cell death (Clapham, 1995). The average [Ca\textsuperscript{2+}]\textsubscript{i} can rise up to several micromolar during stimulation, depending on the cell type, but the resting [Ca\textsuperscript{2+}]\textsubscript{i} has to be maintained at approximately 10-100 nM, which is 20,000-fold lower than the 2 mM Ca\textsuperscript{2+} concentration found extracellularly. Since Ca\textsuperscript{2+} cannot be metabolized like other second-messenger molecules, cells possess complex mechanisms to keep cytosolic Ca\textsuperscript{2+} at a low level and to maintain Ca\textsuperscript{2+} within intracellular pools. Scores of Ca\textsuperscript{2+}-binding proteins have been reported so far (Table 2), some of which change their conformation upon binding Ca\textsuperscript{2+} and modulate effector molecules such as enzymes and ion channels, or trigger second-messenger pathways, whereas others bind Ca\textsuperscript{2+} simply to lower free Ca\textsuperscript{2+} levels as its concentration increases, such as parvalbumin and calsequestrin (Weinman, 1991).

Ca\textsuperscript{2+} pumps such as the plasma membrane Ca\textsuperscript{2+}-ATPase, the sarcoplasmic or endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) in the ER membrane, and exchangers such as the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger located in the plasma membrane, have been found to transport Ca\textsuperscript{2+} ions out of the cytosol into the ER stores or extracellular space. Plasma membrane Ca\textsuperscript{2+}-ATPase was first detected in red blood cells (Schatzmann, 1982), and was later found to be present in all eucaryotic cells. It can pump Ca\textsuperscript{2+} to the extracellular space
Table 2. Examples of Ca\textsuperscript{2+}-binding proteins and their function* 

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>InsP\textsubscript{3} Receptor</td>
<td>Effector of intracellular Ca\textsuperscript{2+} release</td>
</tr>
<tr>
<td>Ryanodine receptor</td>
<td>Effector of intracellular Ca\textsuperscript{2+} release</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-ATPase</td>
<td>Pump of Ca\textsuperscript{2+} across membranes</td>
</tr>
<tr>
<td>Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger</td>
<td>Effector of the exchange of Ca\textsuperscript{2+} for Na\textsuperscript{+} across the plasma membrane</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel</td>
<td>Effector of membrane hyperpolarization</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>Ubiquitous protein kinase</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Ubiquitous modulator of protein kinases and other enzymes</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>Regulator of muscle contraction</td>
</tr>
<tr>
<td>Calpain</td>
<td>Protease</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>Ca\textsuperscript{2+} buffer/modulator of nuclear hormone receptor, Ca\textsuperscript{2+}-dependent chaperone</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>Ca\textsuperscript{2+} buffer in SR lumen</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Ca\textsuperscript{2+} buffer in cytoplasm</td>
</tr>
</tbody>
</table>

* Adapted from Clapham, 1995.
against a $10^4$-fold concentration gradient, using energy from ATP hydrolysis (Pedersen and Carafoli, 1987a, 1987b; Inesi and Kirtley, 1992). The Na$^+$.Ca$^{2+}$ exchanger catalyzes the countertransport of three Na$^+$ for one Ca$^{2+}$. It is a Ca$^{2+}$ extrusion mechanism and requires the energy of the Na$^+$ gradient produced by the Na$^+$ pump. In some tissues such as kidney, smooth muscle, brain, and especially heart, the exchanger activity is extremely high and plays a key role in controlling Ca$^{2+}$ efflux (Khananshvili, 1998; Reeves, 1998; Blaustein and Lederer, 1999). SERCA is a Ca$^{2+}$ pump that is widely distributed within the ER or SR of most cells (Lytton, et al., 1992). The protein helps to maintain a low cytoplasmic Ca$^{2+}$ concentration and is also important for keeping Ca$^{2+}$ within the ER.

The replenishing of intracellular Ca$^{2+}$ stores is critical because intraluminal Ca$^{2+}$ provides the source of cytosolic Ca$^{2+}$ signals and its release triggers Ca$^{2+}$ entry across the plasma membrane (Putney, et al., 1990; Randriamampita, et al., 1993). The Ca$^{2+}$ content of the SR or ER also controls the sensitivity of the RyR and InsP$_3$R release channels (Pozzan, et al., 1994; Bezprozvanny, et al., 1991). In addition, the folding and assembly of nascent proteins in the ER appear to be strongly influenced by intraluminal Ca$^{2+}$ levels (Sambrook, et al., 1990; Kuznetsov, et al., 1992; Lodish, et al., 1992). It was found that two important ER chaperones, calnexin and calreticulin, are both Ca$^{2+}$-binding proteins (Helenius, et al., 1997). In the absence of Ca$^{2+}$, their abilities to facilitate protein folding and assembly are both inhibited. Furthermore, recent studies have demonstrated that the Ca$^{2+}$ content of the ER exerts a profound control over cell growth and the progression of cells through the cell cycle. For example, it was observed that depletion of Ca$^{2+}$ pools using Ca$^{2+}$ pump inhibitors causes DDT1MF-2 cells to enter a stable growth-arrested G$_0$-like state (Ghosh, et al., 1991; Short, et al., 1993).
B. SERCA Ca$^{2+}$ pumps: their function and structure

The SERCA Ca$^{2+}$-ATPases are transmembrane proteins with molecular mass around 110 kDa that bind Ca$^{2+}$ with high affinity and transport it from the cytoplasm to the luminal spaces of the SR/ER (Inesi, et al., 1980). The enzyme is critical for regulating the resting cytoplasmic Ca$^{2+}$ levels as shown by treatment of cells with the Ca$^{2+}$-ATPase inhibitor, thapsigargin, which results in an increase in [Ca$^{2+}$]$_i$ (Thastrup, et al., 1990). On the other hand, SERCA proteins are also important in maintaining intraluminal Ca$^{2+}$ of ER and SR, as demonstrated by the Ca$^{2+}$ pump inhibitors, thapsigargin or 2,5-di-tertbutylhydroquinone, which cause depletion of Ca$^{2+}$ pools. Also, SERCA Ca$^{2+}$ pumps have a significant effect in regulating the complex dynamics of Ca$^{2+}$ signals. For example, Camacho and her colleagues showed that overexpression of SERCA1 in Xenopus oocytes increased the frequency of InsP$_3$-induced Ca$^{2+}$ waves (Camacho and Lechleiter, 1993).

Three differentially expressed genes encode SERCA proteins. Two alternatively spliced species, SERCA1a and SERCA1b, are expressed in fast-twitch skeletal muscles in a developmentally controlled manner. The SERCA2 gene also gives rise to two distinct alternatively spliced transcripts, encoding protein isoforms SERCA2a and SERCA2b. SERCA2a is expressed in cardiac and slow-twitch striated muscles, whereas SERCA2b is ubiquitously expressed. The SERCA3 gene has a selective tissue distribution, being most abundantly expressed in the large and small intestine, thymus, and cerebellum and at lower levels in the spleen, lymph node, and lung (Wu, et al., 1995).

It has been reported that there is greater than 70% amino acid identity among all the Ca$^{2+}$-ATPase isoforms sequenced from birds and mammals (Burk. et al., 1989; Karin, et al., 1989; Cambell, et al., 1991). Since sequence identity is high, studies of structure and function done on SERCA1 are thought to be applicable to all three SERCA gene products. The predicted secondary structure of the SERCA1a and SERCA2a protein is believed to
FIG. 1. A cartoon illustration of the structure of the SERCA \( \text{Ca}^{2+} \) pump. Cylinders correspond to the transmembrane helixes. The residues involved as ligands in the calcium binding sites are indicated in blue, the position of the phosphorylated residue, D351, as well as residues involved in ATP binding are shown in red.
include ten transmembrane helices and this prediction has gained experimental support (Figure 1) (MacLennan, et al., 1985; Matthews, et al., 1990; Clarke, et al., 1990). It has been shown that the N and C termini of the protein reside on the cytoplasmic side, and the M7/M8 loop in the luminal side, of the membrane from well controlled experiments with proteases, antibodies, and sulphhydryl labels (Moller, et al., 1996).

Site-directed mutagenesis has been used to implicate four transmembrane helices (M4, M5, M6 and M8) in forming the Ca^{2+} binding site (Clarke, et al., 1989). Two Ca^{2+} binding sites have been defined and a side-by-side sites model has been suggested (MacLennan, et al., 1997). The binding of Ca^{2+} to site I leads to cooperative binding to site II and occupation of both sites is required for the phosphorylation from ATP. The initial mutation screen identified E309 in M4, E771 in M5, N796, T799 and D800 in M6 and E908 in M8 as Ca^{2+} liganding residues (Figure 1).

The large cytoplasmic domain is formed by two main segments of sequence (Figure 1). One segment of about 130 residues, links M2 to M3. Another segment has about 440 residues, including the phosphorylation site D351, and links M4 to M5. Mutagenesis combined with protein chemistry studies have identified the involvement of G626, D627, F487, R489 and K492 of the large cytoplasmic domain in both catalytic and regulatory ATP binding (Maruyama and MacLennan, 1988; McIntosh, et al., 1996).

Recently, Zhang et al (1998) have provided direct visualization at 8Å resolution of the three dimensional structure of the SERCA1a molecule isolated from rabbit sarcoplasmic reticulum, obtained by cryoelectron microscopy image analysis of decavanadate and thapsigargin stabilized helical tubes. Ca^{2+}-ATPase in these crystals is likely in the E_2 conformation, which is characterized by low-affinity Ca^{2+} binding from the luminal side of the ER. Ten transmembrane α-helices can be discerned, four of which continue into the stalk (Figure 2A). Figure 2B shows one possible arrangement of the transmembrane
FIG. 2. A model for the arrangement of the SERCA transmembrane helices. A. A cartoon illustrating how the ten transmembrane helices (numbered according to Fig. 1) are thought to fit into the electron density map of SERCA (helix 1 is hidden behind helix 6). The yellow helices are arranged in a coiled coil that forms the Ca$^{2+}$ binding cavity (red circle). The position where ATP is thought to bind is also illustrated. B. Proposed arrangement of the ten transmembrane helices shown in A, viewed through the middle of the membrane. Red dots indicate the bound Ca$^{2+}$, with the arrows showing the residues involved in binding them. Adapted from Zhang et al. (1998).
helices. These assignments are based on the most likely connections between stalk helices and transmembrane helices, on cysteine crosslinking experiments, and are consistent with the mutagenesis results that associate M4, M5 and M6 with the two Ca\textsuperscript{2+}-binding sites. The three-stranded coiled coil formed by these helices (see Figure 2A) gives rise to a cavity, extending to the lumen, with a constricted passageway leading to the cytoplasmic surface, thus providing a possible path for Ca\textsuperscript{2+} release to the lumen of the sarcoplasmic reticulum.

C. Molecular mechanism of Ca\textsuperscript{2+} transport

SERCA enzymes are typical P-type ATPases, which are characterized by the formation of a phosphorylated intermediate as part of their catalytic cycle (Inesi, et al., 1992). Two major enzyme conformations, E\textsubscript{1} and E\textsubscript{2}, can be distinguished during the cycle (Figure 3) (Sorensen, et al., 1997). In the E\textsubscript{1} conformation, the Ca\textsuperscript{2+} binding sites are of high affinity and are oriented toward the cytoplasm. In E\textsubscript{2}, the enzyme has a low affinity for Ca\textsuperscript{2+} and the Ca\textsuperscript{2+}-binding sites are oriented luminally.

It was demonstrated by equilibrium experiments that each ATPase monomer binds two Ca\textsuperscript{2+} ions in a cooperative manner, which is attributed to sequential binding (Inesi, et al., 1980). The formation of E\textsubscript{1}P requires that two high affinity Ca\textsuperscript{2+} liganding sites be occupied (see Figure 2). The Ca\textsuperscript{2+}-binding sites are thought to be in a cavity between M4, M5 and M6 that is formed by the precise juxtaposition of Ca\textsuperscript{2+} liganding amino acids located in these helices. Access of Ca\textsuperscript{2+} to the cavity is controlled by interactions between the larger residues close to the cytoplasmic ends of the helices. Inesi (1987) showed that the two Ca\textsuperscript{2+} binding sites were sequentially accessible from the cytoplasm in a crevice with a deep site and a superficial site. The deep site must be first occupied by Ca\textsuperscript{2+}, leaving
FIG. 3. Reaction scheme for Ca\(^{2+}\) transport by SERCA pumps. The reaction proceeds clockwise: two Ca\(^{2+}\) bind to the E\(_1\) form of the enzyme from the cytoplasmic side (left), and the enzyme is then phosphorylated from ATP. A conformational change converts the enzyme from E\(_1\)P to E\(_2\)P, and at the same time releases the two Ca\(^{2+}\) to the luminal side of the ER (right). The enzyme is dephosphorylated, relaxes from E\(_2\) back into E\(_1\), and then re-enters the reaction cycle.
the superficial site for the second ion to bind. Once two Ca\(^{2+}\) ions are bound, the enzyme is then phosphorylated by ATP at residue D351, residing in the cytoplasmic head of the molecule, leading to the formation of the phosphoenzyme intermediate, E\(_1\)P(Ca)\(_2\). Phosphorylation-induced domain movements are believed to close off cytoplasmic access to the Ca\(^{2+}\) binding sites and shift the enzyme into the E\(_2\)P(Ca)\(_2\) conformation. Such movements may also disrupt the precise placement of the ligands required to form the high affinity sites, so the two Ca\(^{2+}\) ions would be less firmly bound and released to the lumen of the ER or SR. E\(_2\)P is then dephosphorylated, the enzyme reverts to E\(_1\), and the high affinity Ca\(^{2+}\)-binding sites are regenerated before reentering into another cycle.

D. Regulation of SERCA function

The contraction of heart muscle is mediated by Ca\(^{2+}\), which enters the myoplasm through slow Ca\(^{2+}\) channels (DHPR) in the transverse tubule and plasma membrane, triggering Ca\(^{2+}\)-induced Ca\(^{2+}\) release through ryanodine receptors (Catterall, 1991). Heart muscle relaxation is modulated by removal of Ca\(^{2+}\) from the sarcoplasm through the combined actions of plasma membrane Ca\(^{2+}\)-ATPase, plasma membrane Na\(^+\)/Ca\(^{2+}\) exchangers, and SERCA pumps (Carafoli and Longoni, 1987). Of these proteins, the cardiac SERCA protein, SERCA2a, plays the predominant role in Ca\(^{2+}\) removal and reloading of the SR stores. It is regulated by phospholamban (PLN), a 52-amino acid integral membrane protein of the cardiac sarcoplasmic reticulum (Kirchberger, et al., 1974; Tada, et al., 1974). It has been well demonstrated that phospholamban is critically involved in the regulation of cardiac contractility (Tada, et al., 1982; Tada, et al., 1983), and is the principal membrane protein phosphorylated in the heart in response to β-adrenergic stimulation (Lindemann, et al., 1983).
PLN is expressed in cardiac muscle, smooth muscle and slow skeletal muscle, but not in fast skeletal muscle (Jorgensen and Jones, 1987; Ferguson, et al., 1988; Jorgensen and Jones; 1986). The protein is a substrate of cAMP-dependent protein kinase (Tada, et al., 1982; Tada, et al., 1983), Ca²⁺-calmodulin-dependent protein kinase (Jones, et al., 1981), and protein kinase C (Iwasa, et al., 1984; Movsesian, et al., 1984). PLN has been predicted to contain three domains (Figure 4). Domain Ia, residues 1-20, contains 6 charged residues and has a net positive charge. Phosphorylation of S16 and T17, which alters the net charge of domain Ia, disrupts the inhibitory function of PLN (Simmerman, et al., 1986; James, et al., 1989). Domain Ia is believed to be partly helical. Domain Ib, residues 21-30, is polar and relatively unstructured (Mortishire-smith, et al., 1995), alternatively, it has been suggested that it might form a β-sheet structure at the membrane interface (Tatulian, et al., 1995). Domain Ib contains only one positive charge in the form of R25 in most species, whereas in human, K replaces N27 resulting in 2 positive charges (Fujii, et al., 1991). Domain II, residues 31-52, is neutral, very hydrophobic, and forms a transmembrane helix. In SDS-polyacrylamide gel electrophoresis, PLN mobility corresponds to the mass of a homopentamer, which is around 25 KDa, but boiling in SDS reduces the apparent mass to that of a monomer or intermediate oligomers (Jones, et al., 1985).

Earlier studies of the interaction between phospholamban and the Ca²⁺-ATPase (Tada and Katz, 1982, Inui, et al., 1986; Suzuki and Wang, 1986; Chiesi and Schwaller, 1989; Xu and Kirchberger, 1989; Kim, et al., 1990; Sasaki, et al., 1992) have provided clear evidence that there is direct interaction between the two proteins, and that this interaction is reversible. It has been demonstrated that a site of functional interaction between the two proteins lies in the phosphorylation domain of the Ca²⁺-ATPase (James, et al., 1989). Also, PLN or PLN mutants have been coexpressed with SERCA2a or
FIG. 4. A model for the inhibitory mechanism of PLN on SERCA2a activity. The three domains of PLN are indicated, on the lower left. An interaction within the membrane between the PLN monomer and SERCA draws the enzyme into the E2 conformation, and inhibits activity. This interaction can be disrupted by phosphorylation of PLN, which disturbs contacts in the cytoplasmic domain (upper left). Ca^{2+} binding to the transport sites draws the enzyme into the E1 conformation, and also disrupts the PLN interaction (upper right).
SERCA2a mutants to define sites of interaction between PLN and SERCA2a (Toyofuku, et al., 1994; Kimura, et al., 1996; Kimura, et al., 1997). Through chimera formation and site-directed mutagenesis, the cytoplasmic interaction sites were identified as charged and hydrophobic amino acids in PLN domain Ia and as amino acids Lys-Asp-Asp-Lys-Pro-Val402 in SERCA2a (Toyofuku, et al., 1994). In 1996, Kimura et al. found that inhibitory interactions were observed when only PLN residues 28-52, containing 3 domain Ib residues plus all of the domain II transmembrane residues, were coexpressed with SERCA2a. They suggested that the inhibitory interaction site lies entirely in the transmembrane sequences of PLN and SERCA2a, but that disruption of noninhibitory cytoplasmic interactions through long-range coupling, can disrupt the membrane sites also.

Alanine-scanning mutagenesis has shown that the inhibitory interaction site in the transmembrane sequence of PLN lies on a helical face that must interact with one or more transmembrane helices in SERCA2a, whereas the other helical face is involved in PLN pentamer formation (Kimura, et al., 1997). Gain of function with PLN mutants that promote PLN depolymerization was observed, and it was found that the most inhibitory mutants of PLN are devoid of pentamer. MacLennan and coworkers concluded that the pentamer represents a less active or inactive reservoir of subunits, and that the PLN monomer is the functional, inhibitory form (Kimura, et al., 1997). Finally, a model of the interaction has been suggested (Figure 4), where PLN monomers are the functional form, and their dissociation from pentamers is required for SERCA2a inhibition. The degree of inhibition is dependent upon the concentration of the inhibited PLN monomer-SERCA2a heterodimeric complex. The concentration of this inhibited complex is in turn determined by both the dissociation constant (Figure 4, K_d1) for the PLN pentamer and the dissociation constant (Figure 4, K_d2) for the PLN/SERCA2a heterodimer. Regulation is superimposed by phosphorylation of PLN or by Ca^{2+}-binding to the membrane transport sites, both of which disrupt the PLN-SERCA2a complex.
E. The C-terminal tail in SERCA2a/b and its functional importance

As described previously, the SERCA2 gene encodes two alternatively spliced protein isoforms: SERCA2a and SERCA2b. SERCA2a is richly expressed in slow-twitch and cardiac muscles, whereas SERCA2b is ubiquitously expressed (Wu, et al., 1995). SERCA2a and SERCA2b have identical primary sequences up to amino acid 993, but differ at their C-terminal ends. As a result of alternative splicing, the last four residues of SERCA2a are replaced with an extended and hydrophobic sequence of 49 amino acids in SERCA2b (Lytton, et al., 1988), which includes a glycosylation consensus sequence-N1035FS. The topology of SERCA2a is believed to be identical to the fast-twitch Ca\textsuperscript{2+}-ATPase (SERCA1) with 10 membrane-spanning domains. According to hydropathy analysis, the extended tail in SERCA2b is predicted to encode an eleventh transmembrane segment (or TMS) (Figure 5) (Lytton and MacLennan, 1988; Gunteski-Hamblin, et al., 1988).

Two independent experiments have demonstrated clearly that the COOH-terminal of SERCA2b is inside the ER. In 1992, epitope mapping was used by Campbell et al. to address the orientation of the SERCA2b C-terminal tail. The human c-myc epitope, a 10 amino acid sequence recognized by monoclonal antibody 9E10, was added onto the carboxyl termini of SERCA2a and SERCA2b. The added epitopes did not appear to disrupt topology as judged from unaltered Ca\textsuperscript{2+} transport activity. With the combination of immunocytochemical studies, and a procedure to permeabilize the plasma membrane selectively with the bacterial toxin streptolysin-O (SLO), they demonstrated that SERCA2a and SERCA2b have their carboxyl termini on opposite sides of the ER membrane, with SERCA2a’s is in the cytosol and SERCA2b’s is in the lumen (Campbell, et al., 1992). Later, in 1995, an in vitro translation method was used to determine the orientation of the SERCA2b C-termini (Bayle, et al., 1995). A fusion vector containing signal anchor
**FIG. 5. Expanded view of the SERCA2a and SERCA2b C-termini.** The sequence of both SERCA2a and SERCA2b following amino acid 993 is illustrated. SERCA2b is shown to traverse the membrane an extra time, as M11, and the potential glycosylation site (Asn 1035) in the lumen of the ER is highlighted in grey.
sequences and stop transfer sequences was used to determine the presence or absence of membrane insertion sequences. Assembly of polytopic membrane proteins that contain an even number of TMS is believed to require the presence and sequential insertion of topogenic signals into the membrane of the ER. Odd numbered segments act as signal anchor sequences, and even numbered segments act as stop transfer sequences (Friedlander and Blobel, 1985). It was found that the hydrophobic sequence representing the 11th putative TMS of the SERCA2b acted as both a signal anchor and a stop transfer sequence. This result indicated that the SERCA2b C-terminal tail resided in the ER lumen, which is consistent with the finding of Campbell et al (1992).

Functional comparisons were studied between different isoforms of SERCA in a COS-1 cell expression system (Lytton, et al., 1992). SERCA2b was found to have a two-fold lower turnover rate for both Ca\(^{2+}\) transport and Ca\(^{2+}\)-dependent ATP hydrolysis than SERCA2a. In addition, they found that the Ca\(^{2+}\)-dependency of Ca\(^{2+}\)-uptake activity for SERCA2b was slightly higher than that of SERCA2a. The functional differences between the two SERCA2 gene products were confirmed by Casteels' group (Verboomen, et al., 1994), using Ca\(^{2+}\)-uptake. These will be referred to as the SERCA2b and SERCA2a "phenotypes", respectively.

To investigate the functional importance of subdomains in the SERCA2b tail, Casteels' group (Verboomen, et al., 1994) constructed three SERCA2b mutants (mutant I, II, III) with truncations of 12, 31 or 49 amino acids, respectively. The mutants and wild type SERCA2 cDNAs were transfected into COS-1 cells and analyzed for functional differences. These experiments revealed that the turnover rate (v/EP), defined as the initial rate of Ca\(^{2+}\) uptake (v) divided by the steady-state level of phosphorylated enzyme intermediate (EP), which is a measure for the level of active Ca\(^{2+}\)-ATPase, for all three truncated SERCA2b mutants, was higher than for SERCA2b, and approximately similar to that of SERCA2a. They also demonstrated that all three mutants had the same Ca\(^{2+}\)-
dependence for Ca\textsuperscript{2+}-uptake activity and ATP-hydrolysis rate as SERCA2a, which is different than that of wild type SERCA2b. In addition, they also tested the vanadate-sensitivity of SERCA2a and SERCA2b. Vanadate is thought to be a structural analogue of enzyme bound phosphate which can inhibit the SERCA activity by associating with the molecule in the E\textsubscript{2} conformation and preventing further progress through the catalytic cycle. The results revealed that each of the mutants resembled SERCA2a (K\textsubscript{0.5} = 0.01 mM) and differed from SERCA2b (K\textsubscript{0.5} = 0.1 mM). It was suggested that SERCA2b had a higher apparent affinity for Ca\textsuperscript{2+} due to having a larger population in the E\textsubscript{1} than in the E\textsubscript{2} conformation compared to SERCA2a and the three mutants. Since all three truncated versions of SERCA2b acquire the same properties as SERCA2a with respect to their Ca\textsuperscript{2+} affinity, vanadate-sensitivity and turnover rate, the authors concluded that the last 12 amino acids of SERCA2b are of particular importance to its function.

F. Calreticulin (CRT)

CRT is a protein first purified in the early 1970s by MacLennan and his colleagues from skeletal muscle sarcoplasmic reticulum and named high affinity Ca\textsuperscript{2+}-binding protein (Ostwald and MacLennan, 1974). Subsequently, this protein was named calreticulin and has been shown to be ubiquitously expressed and highly conserved among different species, and located predominantly in the ER/SR (Michalak, et al., 1992; Michalak, et al., 1991; Opas, et al., 1991; Tharin, et al., 1992).

F.1 The structural and function of CRT

Structural predictions for CRT suggest that it has three functional domains: an N-terminal region (N-domain), which is the most conserved domain across different species; a proline-rich region with internal repeat sequences (P-domain); and an acidic C-domain that terminates with the ER retrieval signal (KDEL) (Michalak, et al., 1997). The P-domain
has a high affinity but low capacity binding site for \( \text{Ca}^{2+} \) (Baksh and Michalak, 1991), whereas the C-domain binds \( \text{Ca}^{2+} \) with low affinity and high capacity (Michalak, et al., 1992).

CRT binds 1 mole of \( \text{Ca}^{2+} \) per mole of protein with micromolar affinity and more than 20 moles of \( \text{Ca}^{2+} \) per mole of protein with millimolar affinity (Michalak, et al., 1992). Using \(^{45}\text{Ca}^{2+}\) uptake, an increased amount of intracellularly stored \( \text{Ca}^{2+} \) has been shown in transiently transfected calreticulin-overexpressing HeLa cells (Bastianutto, et al., 1995) as well as in stably transfected mouse L fibroblasts overexpressing calreticulin (Mery, et al., 1996). Numerous functions have been reported for CRT: it acts as a molecular chaperone in facilitating the folding and assembly of nascent membrane and secreted proteins (Helenius, et al., 1997); it contributes to the regulation of cell adhesiveness (Dedhar, 1994; Coppolino, et al., 1997; Opas, et al., 1996); it modulates steroid-sensitive gene expression (Burns, et al., 1994; Dedhar, 1994); furthermore, it is involved in the regulation of intracellular \( \text{Ca}^{2+} \) signaling (Liu, et al., 1994; Bastianutto, et al., 1995; Mery, et al., 1996).

One of the most striking aspects of calreticulin function is its preference for binding to Asn-linked glycoproteins. The basis for this selectivity arises from the fact that calreticulin is a lectin that binds the oligosaccharide processing intermediate \( \text{Glc}_{1}\text{Man}_{9}\text{GlcNAC}_{2} \) (Ware, et al., 1995; Spiro, et al., 1996; Hammond, et al., 1994; Hebert, et al., 1995). That the binding involves a direct oligosaccharide-protein interaction, with a specificity for monoglucosylated oligosaccharides, has been confirmed \textit{in vitro} (Ware, et al., 1995; Spiro, et al., 1996). It was observed that if formation of the \( \text{Glc}_{1}\text{Man}_{9}\text{GlcNAC}_{2} \) oligosaccharide is blocked with tunicamycin, or if production of the \( \text{Glc}_{1}\text{Man}_{9}\text{GlcNAC}_{2} \) species is prevented by treatment with the glucosidase inhibitors castanospermine or deoxynojirimycin, the binding of calreticulin to the most of the proteins is inhibited (Nauseef, et al., 1995; Peterson, et al., 1995; Hammond, et al., 1994; Ou, et
al., 1993). CRT associated with nascent glycoproteins in cycles of binding and release regulated by glucosidase II, which removes the single glucose residue from the Glc1Man9GlcNAc2 oligosaccharide, as well as by UDP-glucose: glycoprotein glucosyltransferase, which reattaches it (Hebert, et al., 1995). The interaction promotes efficient folding of the substrate proteins and prevents their degradation and premature oligomerization (Hammond and Helenius, 1994; Kearse, et al., 1994; Romagnoli and Germain, 1995; Hebert, et al., 1996; Vassilakos, et al., 1996). Also, it plays a critical role in the quality control process which prevents export of immature proteins from the ER to the Golgi complex and beyond (Hammond, et al., 1994; Jackson, et al., 1994; Rajagopalan, et al., 1994).

F.2 The relationship of CRT and SERCA

It was observed by Camacho and Lechleiter (1993) that the release of ER Ca2+ through activated InsP3Rs leads to complex spatiotemporal patterns of repetitive Ca2+ waves. They found that expressing SERCA1 increased the frequency of the Ca2+ waves presumably by speeding up cytoplasmic clearing and refilling of the stores. In 1995, Camacho and Lechleiter examined the role of CRT in InsP3-mediated Ca2+ waves. They found that, similar to SERCA1, when SERCA2b was overexpressed in Xenopus oocytes high frequency repetitive Ca2+ waves were induced by InsP3. CRT had dramatic effects on these Ca2+ waves when it was co-expressed with SERCA2b. CRT did not affect the peak amplitude of InsP3-mediated Ca2+ release, but did reduce the occurrence and frequency of repetitive Ca2+ wave activity. Through deletion analysis the authors identified that the inhibitory effect was mediated via the high affinity low capacity Ca2+-binding P-domain of CRT. More recently, Camacho and her colleagues (John, et al., 1998) reported different Ca2+ wave properties between SERCA2a and SERCA2b-overexpressing oocytes.
Interestingly, they found that CRT only inhibits Ca\(^{2+}\) oscillations when coexpressed with SERCA2b, but not when coexpressed with SERCA2a. They then tested the hypothesis that the functional effects of CRT on Ca\(^{2+}\) waves are mediated by interaction with SERCA2b via an oligosaccharide attached to the putative glycosylation site present in the unique C-terminal sequence extension. Pharmacological inhibition of glucosidases, expected to prevent the interaction of CRT with oligosaccharides (see above), abolished the effect of calreticulin on Ca\(^{2+}\) waves induced by SERCA2b. In addition, they demonstrated that mutation of residue N1035 of SERCA2b, the putative oligosaccharide acceptor site, abolished the effects of CRT on SERCA2b induced Ca\(^{2+}\) waves.

Numerous questions have been raised by these findings: Does CRT influence SERCA2b function directly? Do CRT and SERCA2b physically associate with one another? If so, how? Does the interaction require the presence of Ca\(^{2+}\)? Does CRT also interact with other SERCA isoforms? These questions have resulted in the formation of a specific hypothesis: that CRT specifically interacts with an oligosaccharide chain present on N1035 of the SERCA2b luminal tail by virtue of its Ca\(^{2+}\)-dependent lectin activity, resulting in inhibition of SERCA2b activity. This hypothesis explains the influence of overexpressed CRT on Ca\(^{2+}\) waves observed in *Xenopus* oocytes, as well as the lower turnover rate of SERCA2b observed in COS cells, due to endogenously expressed CRT.

G. Research Goals

The objective of my research was to address the structural cause for the functional difference observed between the two isoforms of SERCA2, and to explore possible molecular mechanisms underlying this difference. A HEK-293T cell expression system was used to examine the biochemical properties of the two SERCA2 isoforms. The first hypothesis tested was that their functional difference was due to the interaction of CRT
with an oligosaccharide attached to N1035 in the SERCA2b C-terminal tail. A SERCA2b mutant with N1035 changed to A (denoted Mut2b), which destroys the putative glycosylation site, was created to examine its influence on SERCA2b activity. Immunoprecipitation was then used to investigate the interaction between CRT and SERCA2b.

The second hypothesis was that critical amino acids involved in underlying the functional difference between SERCA2a and SERCA2b could be identified by progressive deletion mutants. Constructs with truncations from the C-terminal end of SERCA2b of increasing length (3, 6, 9 and 12 amino acids) were prepared and transfected into HEK-293T cells. Microsomes were isolated from cells overexpressing wild type SERCA2a, SERCA2b, or the SERCA2b mutants, and their ATPase activity compared.

Our third hypothesis was that the orientation of the C-terminal tail could also play an important role in SERCA2b's activity. A construct with the addition of an "L" between the two Ls of the central sequence VLLI in the last transmembrane region of SERCA2b was prepared. The insertion of the "L" was expected to change the length of the α-helix and also to rotate the orientation of the luminal tail by ~100°. The construct was transfected into HEK-293T cells, and microsomes isolated to investigate changes in SERCA2b activity.

The last hypothesis of this project was that the C-terminal tail may interact with other region(s) of SERCA2b, thereby inhibiting activity. A peptide consisting of the last 12 amino acids of SERCA2b, plus a Cys residue at the N-terminal end, was synthesized, and the influence of the peptide on SERCA2a activity was investigated by incubating the peptide with solubilized SERCA2a.

The findings of these studies will contribute to our understanding of Ca\(^{2+}\) signalling and of the difference in mechanism between the two isoforms of the SERCA2 gene.
CHAPTER TWO
EXPERIMENTAL PROCEDURES

All molecular biology procedures were conducted essentially according to published protocols (Ausubel, et al., 1998; Sambrook, et al., 1989) unless otherwise indicated. Chemicals were of the highest available molecular biology grade from Fisher, BDH, or Sigma or as listed.

A. Oligonucleotide-directed Mutagenesis

Constructs encoding human SERCA2a and SERCA2b in the mammalian expression vector pcDNA3.1 were previously prepared in the Lytton laboratory by Stephen Leach. A multi-step strategy was designed that allowed replacement of only a small fragment of SERCA2b by polymerase chain reaction (PCR) to create the desired mutations (Figure 6). The fragment of 1767 basepairs between unique BamH I and EcoR V sites encoding part of the SERCA2b C-terminal sequence as well as part of the pcDNA3.1 vector sequence, was ligated into pBluescript II SK(-), generating construct SK+2B. The codon encoding the potential glycosylation site, Asn1035, was mutated into Ala, the amino acids Ser1031, Thr1034, Ser1037, Phe1010 were each mutated into a stop codon, and an amino acid Leu (L) was inserted between the two Ls of the VLLV sequence of the last transmembrane segment, generating constructs with point mutation N1035A and deletions from the C-terminus of increasing length 3, 6, 9, 12, as well as an insertion of L. Each construct was prepared using synthetic primers (Table 3) and a two step PCR protocol to amplify a small segment encoding the C-terminus of the protein. The products were then ligated into the SK+2B construct using unique Sph I and EcoR V cutting sites. The fragment containing the desired mutation was subcloned back into the parent construct.
FIG. 6. The multi-step strategy for mutation. The 535 basepair segment between Sph I and EcoR V of the construct pcDNA 3.1(+)-SERCA2b was amplified by PCR using synthetic primers carrying the point mutation (primers “F” and “R” combined with “Forward” and “Reverse” primers, respectively, as shown in the upper line). The BamH I–EcoR V region was subcloned into pBluescript SKII(−) creating construct “SK+2b”. Both the PCR product and SK+2b were digested with Sph I and EcoR V and ligated together to create SK+2b-Mut. The BamH I–EcoR V region of pcDNA 3.1(+)-SERCA2b was then replaced with the BamH I–EcoR V region of SK+2b-Mut.
Table 3. Sequence of oligonucleotide primers*

<table>
<thead>
<tr>
<th>Name</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward-SERCA Primer</td>
<td>5'-CACCAATCCTGCTCGTCTCG-3'</td>
</tr>
<tr>
<td>Reverse-SERCA Primer</td>
<td>5'-CTCCAGCTGCCATAGCTATT-CG-3'</td>
</tr>
<tr>
<td>Primer F (delete 3 AA)</td>
<td>5'-CTTTTA GCGATATGTAATGGTTCTTG-3'</td>
</tr>
<tr>
<td>Primer R (delete 3 AA)</td>
<td>5'-TCAAGACCATTACATCAGCTAAAG-3'</td>
</tr>
<tr>
<td>Primer F (delete 6 AA)</td>
<td>5'-CTAACTTTAGATATGTTCTGTGTC-3'</td>
</tr>
<tr>
<td>Primer R (delete 6 AA)</td>
<td>5'-GACCAAGACATATCTCAAAAGTTAG-3'</td>
</tr>
<tr>
<td>Primer F (delete 9 AA)</td>
<td>5'-CTATAGCAGACTGAAACTTTTAGC-3'</td>
</tr>
<tr>
<td>Primer R (delete 9 AA)</td>
<td>5'-GCTAAAGTTTCAAGGTGCTATAG-3'</td>
</tr>
<tr>
<td>Primer F (delete 12 AA)</td>
<td>5'-CTGGGTCTATTGAAACAGACACTAA-3'</td>
</tr>
<tr>
<td>Primer R (delete 12 AA)</td>
<td>5'-TTAGTGTCTGTTCAATAGACCCAG-3'</td>
</tr>
<tr>
<td>Primer F (N1035A)</td>
<td>5'-GCACAGACACTGCCATTTAGCAGATAG-3'</td>
</tr>
<tr>
<td>Primer R (N1035A)</td>
<td>5'-CATATCGCTTAAAGGCTGTGCTTGC-3'</td>
</tr>
<tr>
<td>Primer F (Insert L)</td>
<td>5'-GTGTTGTCGTCTACTACAAGCTAATGC-3'</td>
</tr>
<tr>
<td>Primer R (Insert L)</td>
<td>5'-CATTATGAGCAAGCAGCAAAACG-3'</td>
</tr>
<tr>
<td>Forward Primer HumanCRT</td>
<td>5'-GGGTTACCGGCAAGGCTGTGCTATCC-3'</td>
</tr>
<tr>
<td>Reverse Primer HumanCRT</td>
<td>5'-GCTCTAGATCTCTACAGCTCGTCTCTTG-3'</td>
</tr>
<tr>
<td>Forward Primer (2b entire tail)</td>
<td>5'-CCGGTACCAGGAGACTACAAGGATGATG ATGACAAGGGTTAAAGGTGTGTCAGGCT-3'</td>
</tr>
<tr>
<td>Reverse Primer (2b entire tail)</td>
<td>5'-CGGATATCTCAAGACAGACATATCGCTAA-3'</td>
</tr>
</tbody>
</table>

* The primers labeled “F” and “R” were used in conjunction with the “Reverse SERCA” and “Forward SERCA” primers, respectively, to create the various mutant constructs. See Figure 6 for explanation. The underlined nucleotides indicate the mutated nucleotides, which are different from the original.
using the BamH I and EcoR V sites. The presence of the desired mutation was confirmed by sequencing from the Sph I site to the end of the SERCA2b cDNA sequence.

B. Synthesis of human calreticulin

The human calreticulin (CRT) cDNA was obtained using reverse transcription-coupled polymerase chain reaction (RT-PCR). First-strand cDNA was obtained from oligo (dT)-primed Jurkat-T cell total RNA using RNaseH (-) Mu-MLV reverse transcriptase (Superscript II; Life Technologies). PCR primers (Table 3) encompassing start and stop codons were synthesized based on the human CRT sequence (Genbank Accession: M84739). The products were then ligated into the pCDNA 3.1(+) vector. The absence of PCR errors was determined by DNA sequencing.

C. Synthesis of construct “2b entire tail”

A construct denoted “2b entire tail” encoding the last 49 amino acids of SERCA2b plus an N-terminal FLAG-epitope (DYKDDDDK) was synthesized by PCR (primers shown in Table 3). The PCR products were ligated into the pCDNA 3.1(+) vector followed by sequencing to ensure the absence of PCR errors.

D. Cell culture and Transfection

Tissue culture reagents were purchased from Life Technologies, Inc. HEK-293T cells, obtained from Ron Kopito’s lab at Stanford, have been transformed with SV40 large T antigen. They behave quite differently from HEK-293T cells that originate from ATCC. First, HEK-293T cells do not stick very tightly to plastic tissue culture plates. Second, HEK-293T cells can be transfected very efficiently using the Ca\(^{2+}\) phosphate method, and produce a large amount of recombinant protein. Cells were maintained at 37°C under 5% CO\(_2\) in high glucose Dulbecco’s modified Eagle’s medium which was supplemented with
2 mM additional L-glutamine, 1% MEM non-essential amino acids, 10% fetal bovine serum, and 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were passaged just prior to confluence twice per week, using a split ratio of 1:15 on Mondays and 1:20 on Thursdays. Cells at 40-60% confluence in 10-cm dishes were transfected using Qiagen column-purified plasmids and a standard Ca\(^{2+}\) phosphate precipitation protocol with BES buffer as described previously (Toyofuku, et al., 1994). Briefly, 10 μg of DNA was diluted into a final volume of 0.45 ml with water, followed by the addition of 50 μl of 2.5 M CaCl\(_2\). This solution was then added dropwise to 0.5 ml of 2XBES buffer, which contained 280 mM NaCl, 50 mM BES, 1.5 mM Na\(_2\)HPO\(_4\), pH 7.0, using a pipetting-aid to provide a steady stream of small bubbles from the bottom of the tube for mixing. The solution usually became slightly cloudy in 5 minutes. 1 ml of this cloudy solution was added dropwise to each plate. The plates were then swirled gently and returned to the incubator.

### E. Membrane Preparations

A crude, post-mitochondria membrane fraction, termed “microsomes” was obtained from the transfected HEK-293T cells as described previously (Lytton, et al., 1992). Two days after transfection, cells were washed twice with phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH 7.4), harvested in PBS containing 5 mM EDTA, and washed with PBS. Cells were resuspended in 10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl\(_2\) and allowed to swell for 10 min on ice. Aprotinin (10 μg/ml) and phenylmethylsulfonyl fluoride (0.1 mM) were added to prevent protein degradation, and the cells were disrupted with a Dounce homogenizer. The suspension was diluted with an equal volume of a solution containing 0.5 M sucrose, 6 mM β-mercaptoethanol, 40 μM CaCl\(_2\), 10 mM Tris-HCl, pH 7.5, 0.3 M KCl and
centrifuged at 8,000 g for 15 min. The supernatant was adjusted to 0.6 M KCl and centrifuged at 120,000 g for one hour using a Ti75 rotor at 4°C. Finally, the “microsomes” were suspended in solution B (250 mM sucrose, 10 mM Tris–HCl, pH 7.5, 20 μM CaCl2, 150 mM KCl, and 3 mM β-mercaptoethanol). Protein concentration was determined by the dye binding assay of Bradford (1976) using bovine γ-globulin as a standard with the Protein Assay kit from Bio–Rad. The average protein concentration obtained from five 10 cm plates of cells was about 2 mg/ml in a 200 μl total volume.

F. Immunoblotting of Microsomal Proteins

Samples for immunoblotting were dissolved without heating in 2×Laemmli sample buffer containing 4% β-mercaptoethanol, resolved on 9% SDS-polyacrylamide gels according to Laemmli (1970), and electrophoretically transferred to nitrocellulose membranes (Towbin, et al., 1979). The membranes were incubated 30 min with 5% dried milk powder in PBS containing 0.1% Tween (PBS-T) to block non-specific binding.

The membrane was incubated with primary antibody diluted in PBS-T plus 1% milk powder for 1-2 hours at room temperature, then washed three times in PBS-T, followed by incubation for 30 min with horseradish peroxidase-conjugated secondary antibody diluted 1:10,000 in PBS-T plus 1% milk powder, washed again, and developed by enhanced chemiluminescence using the Pierce Supersignal substrates. SERCA expression was detected using rabbit polyclonal anti-SERCA antibody N1 (1:4000) or C4 (2 μg/ml), or mouse monoclonal anti-SERCA2 antibody IID8 (1:1000). The N1 antibody was generated in the Lytton laboratory against the large central cytosolic domain (amino acids 362-705) of rat SERCA2 and is of high titer and specificity toward both SERCA2 and SERCA3 when tested by blotting microsomes from transfected HEK cells. The C4 antibody was prepared against the rabbit SERCA2 fragment, amino acids 17-215, and recognizes all SERCA isoforms (Lytton and MacLennan, 1988; Lytton, et al., 1992). The
IID8 antibody is specific for SERCA2 and was obtained from Affinity BioReagents, Colorado. The expression of CRT was detected using rabbit polyclonal C-terminal chicken anti-CRT (1:1000) prepared against mouse CRT amino acids 399-414 and was also obtained from Affinity BioReagents, Colorado.

G. Immunoprecipitation

For the test of co-immunoprecipitation of SERCA with CRT, 50–80 µg of transfected microsomal protein was solubilized in 1 ml of either 1% (w/v) CHAPS, or 1% (w/v) digitonin in IP buffer (150 mM NaCl, 25 mM TrisCl, pH 7.5, 2.5 mM DTT and plus the protease inhibitors, 0.5 mM Benzamidine, 1 µg/ml leupeptin, 2 µg/ml pepstatin, 0.15 mM phenylmethylsulfonyl fluoride) containing 1 mg/ml of ovalbumin as carrier protein, and either 0.5 mM CaCl₂ or 1 mM EGTA. Samples were incubated on ice for 10–20 min, spun 5 min at maximal speed in the microcentrifuge in the cold room (4°C) to remove particulate material. Samples were “precleared” to remove nonspecifically associated material by adding 100 µl of 20% protein A-Sepharose beads to each sample and incubated at room temperature for 30 min with rocking. Samples were then centrifuged at 4,000 g for 1 min to pellet the protein A beads, and the supernatant was transferred to a clean tube. Polyclonal anti-SERCA antibody ~10 µl (either N1 or C4) was added to each sample and incubated with rocking for 2 hours at 4°C. Finally, 100 µl of 20% protein A beads were added and incubation continued with rocking for 30 min at 4°C. The beads were then collected in the microcentrifuge at 4,000 g for 1 min, and washed 3 times successively with the IP buffer without protease inhibitors and carrier protein, and dissolved in 30 µl of gel loading buffer (which contains Laemmli sample buffer, plus 2% SDS, and 5% β-mercaptoethanol) and incubated 5 min at 50-60°C. The samples were spun down to remove the beads and the supernatant was loaded on the gel.
H. **Determination of SERCA content by “E-P” trapping**

The covalent phosphorylated intermediate of the enzyme was quantified as described before (Maruyama and MacLennan, 1988). 5 μl of HEK-293T cell microsomes (∼2 μg/μl) was added to 40 μl buffer (100 mM KCl, 25 mM MOPS-KOH, pH 7.0, 6.25 mM MgCl$_2$), containing either 5 μl of 1 mM CaCl$_2$ or 10 mM EGTA. A drop (i.e. 100 μg) of 10% bovine serum albumin (BSA) was suspended on the side of the tube as carrier prior to the start of the reaction. The assay was initiated by the addition of 2.5 μl of 40 μM ice-cold γ-[³²P] ATP (1 mCi/ml). The reaction was quenched 10-15 seconds later by the addition of 1 ml of ice-cold 10% trichloroacetic acid (TCA)/1 mM H$_3$PO$_4$. The precipitate was allowed to form for a few minutes on ice and then sedimented in a microcentrifuge for 10 min at 15,000 g in the cold room. The pellet was washed 2 times with TCA/H$_3$PO$_4$ and finally dissolved in 250 μl of 0.1 M NaOH/0.1% SDS, and the incorporated radioactivity was determined using Cherenkov counts in the scintillation counter without adding the scintillation cocktail. Cherenkov radiation is the blue light emitted when a charged particle moves in a transparent medium with a speed greater than that of light in the same medium. It is detected in the tritium channel of a scintillation counter. The specific radioactivity (SA) in the original stock of 40 μM γ-[³²P] ATP (total counts) was determined by counting a diluted aliquot under the same conditions as the dissolved pellet. The calculation of pmol SERCA/mg membrane protein was performed as follows:

$$SA(\text{cpm/pmol ATP}) = \frac{\text{Average(total counts)}}{(\text{ATPconc.Xdilution factorXvolume counted})}$$

$$\text{pmolSERCA/mg} = \frac{(\text{Average (counts from each assay/SA)})}{(\text{protein conc.Xaliquot volume})}$$

I. **Assay of ATPase activity**

ATPase activity was determined at 30°C using an enzyme-coupled spectrophotometric assay (Rossi, et al., 1979). This assay couples the hydrolysis of ATP
to the oxidation of NADH, which can be followed as a decrease in A_340. The molar extinction coefficient for NADH at 340 nm is 6230. As set up, this assay starts with an absorbance of about 2.0. The assay solution contained 120 mM KCl, 25 mM MOPS/KOH pH 7.0, 2 mM MgCl_2, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 1 mM DTT, 0.32 mM NADH, 10 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase, 2 μM ionophore A23187, with either 0.5 mM EGTA or 0.45 mM CaCl_2/0.5 mM EGTA. The assay was performed in a Beckman DU640 spectrophotometer using a six cell cuvette holder thermostatted to 30°C. Quartz cuvettes and reaction buffer (0.3 ml/cuvette) were equilibrated to temperature, and the reaction was started by the addition of microsomal protein (~8 μg/cuvette) with rapid mixing. The reaction was followed for 10 min, the data plotted, and the linear portion of the curve (generally from about 30 sec–1 min [for equilibration, etc.] onward) was used to determine the rate of enzyme activity.

For the Ca^{2+}-affinity assay, different concentrations of free Ca^{2+} were generated using EGTA as a buffer. The EGTA-Ca^{2+} dissociation constant (K_{app}) was corrected for ionic strength (I) and pH according to the data of Harafuji and Ogawa (1980) using the following equation:

\[
\log(K_{app}) = 6.46 - (2\sqrt{I} + \sqrt{I} - 0.4 I) + 2 (pH - 6.8)
\]

The data from experiments measuring the dependence of activity upon Ca^{2+} have been fit by computer to the general cooperative model for substrate (S) activation:

\[
v = V_{\max} [S]^n / (K_{1/2}^n + [S]^n)
\]

where \(V_{\max}\) is the maximum activity reached, \(K_{1/2}\) is the substrate concentration which gives half of \(V_{\max}\), \(n\) is the equivalent to the Hill coefficient. The program MacCurveFit, run on an Apple Macintosh computer, was used for general non-linear curve-fitting, setting \(V_{\max}\) to 100%, and \(n\) to 1.5, corresponding to partial cooperativity between the two Ca^{2+}
binding sites, as reported previously by others (Lytton, et al., 1992; Toyofuku, et al., 1992).

To investigate the effect of the peptide consisting of the last 12 amino acids of the SERCA2b C-termini on SERCA2a activity, the peptide was dissolved in solution B (see "Membrane Preparations", above), pH 8.0, to make a final concentration of 250 μM. Cardiac sarcoplasmic reticulum (CSR) was solubilized in solution B containing 0.4 mg/ml octaethylene glycol monododecyl ether (C₁₂E₈) at a final protein concentration of 0.5 mg/ml. This detergent was chosen because of its ability to solubilize the Ca²⁺-ATPase in an active state (Lund, et al., 1989). The peptide and the microsomes were preincubated either 40 min at 0°C or 5 min at room temperature before the activity measurement.

J. Turnover rates

The molecular turnover rates (turnover number/min) were calculated by dividing Ca²⁺-ATPase activity (pmol/min/mg membrane protein) by the concentration of active enzyme present (pmol/mg membrane protein) determined by covalent "E-P" trapping.

K. Data analysis

The activity comparisons shown in Figure 8 and Figure 12 are means of 4 to 14 measurements performed with independent membrane preparations. Each measurement consisted of triplicate Ca²⁺-ATPase activity assays and triplicate or quadruplicate EP assays to quantify the protein. In Figure 8, turnover number/min was used for activity comparison. In Figure 12, the ratio of activity of each sample relative to wild type SERCA2b determined on the same day for membrane preparations isolated on the same day in parallel was used for activity comparison. The reason that the relative ratio was used rather than the turnover number/min was because in some membrane preparations the turnover number/min was significantly different from other preparations, for reasons that
are unclear. The relative differences between SERCA2a, SERCA2b, and the mutants tested was maintained, however. The significance of functional differences between each SERCA2b mutant and the wild type SERCA2b was performed using a paired, one-tailed t-test, calculated by the program Microsoft Excel.
CHAPTER THREE
RESULTS

The objective in this study was to determine which amino acids in the C-terminal end of SERCA2b were critical in underlying the functional difference between SERCA2a and SERCA2b, and to explore the molecular mechanism of the SERCA2b C-terminus in regulating SERCA2b activity. To accomplish this goal, mutagenesis of specific sites in the SERCA2b tail was combined with expression of the combinator protein, followed by measurement of activity.

A. Expression in HEK-293T cells and protein quantification

The system of transient expression in HEK-293T cells was used for addressing the relation of structure to function for the SERCA enzyme. The reason for this choice was the high efficiency and rapidity compared to stable transfection, as well as the ease and simplicity of the preparations and assays. Expression of transfected molecules in HEK-293T cells is usually 10 fold higher than endogenous levels, therefore, overexpressed SERCA activity can be distinguished easily from the endogenous activity. HEK-293T cells were transfected with expression constructs encoding SERCA2a, SERCA2b, and the mutants to be tested, as well as the empty vector alone as a control. Following transfection, a membrane preparation, enriched for SERCA protein, was isolated, and SERCA expression assessed by a number of methods. Microsomal proteins were separated on SDS-containing Laemmli slab gels, transferred to nitrocellulose, and probed using either the N1 polyclonal antisera against SERCA, the C4 antisera against SERCA, or the IID8 monoclonal antibody (Figure 7). Both SERCA2a and SERCA2b were expressed at high levels compared to control transfected cells. As can be seen on immunoblots, SERCA2b showed some tendency to form dimers on SDS-containing gels, in contrast with
FIG. 7. Immunoblot of SERCA proteins. 5 μg of microsomes, isolated from HEK-293T cells transfected with the indicated constructs, were separated by SDS-PAGE, transferred to nitrocellulose, and probed with I1D8 (diluted 1:1000), N1 (1:4000), or C4 (2 μg/ml) antibodies, as indicated.
SERCA2a. This is believed to be related to the unique extended carboxyl-terminal sequence of SERCA2b. Also, the mobility of SERCA2a is slightly faster than SERCA2b, which indicates the smaller size of the protein (SERCA2a is predicted to be 109.5 kDa, whereas SERCA2b is 114.6 kDa).

Activity of expressed SERCA is determined by the inherent properties of the enzyme and its level of expression. Initially, the level of expressed protein was quantified by immunoblot, using a series of increasing amounts of microsomal protein, and enhanced chemiluminescent detection or fluorescent detection, captured using the Molecular Dynamics fluorescent imager. In addition, enzyme-linked immunoadsorbent assays were tried. These methods were given up because of the difficulty in getting a linear response and the large variation in the data. Finally, the amount of active protein present in the transfected HEK-293T cell microsomes was determined by trapping the covalent phosphorylated intermediate formed during enzyme turnover (E-P) (see Experimental Procedures). Previous experiments have established that 2 µM ATP and 15 s on ice produced maximal incorporation (Maruyama and MacLennan, 1988). Titrations of SERCA1a in skeletal muscle SR were employed in pilot experiments to ensure linearity of the assay. This method has been working consistently well and was employed in the quantification of all SERCA proteins in this project. An important advantage of this technique is that it only traps the proteins that have activity, whereas immunological detection does not discriminate between active and inactive (denatured) proteins.

B. Effect of point mutation N1035A on SERCA2b activity

Based on previous publications (Camacho, et al., 1995; John, et al., 1998), our first hypothesis was that changing only one residue Asn1035 within the final 12 amino acids, which forms the potential glycosylation site, to Ala would be sufficient to convert the SERCA2b phenotype to a SERCA2a one. We also predicted that calreticulin (CRT) would
FIG. 8. **Ca$$^{2+}$$-ATPase activity comparison.** A, A representative immunoblot is shown. 2 μg of HEK-293T cell microsomes, transfected with the indicated SERCA constructs, were separated by SDS-PAGE, transferred to nitrocellulose, and probed with IID8 anti-SERCA antibody. B, Ca$$^{2+}$$-ATPase activity comparison between SERCA2a, SERCA2b and Mut2b (N1035A). The average ± standard error of the mean is shown for between four and nine determinations. The 2b and M2b values were found to be significantly different from 2a, using a one-tailed paired t-test, with P<0.03.
physically associate with SERCA2b and that this interaction would serve to inhibit the enzyme activity. To test our hypothesis, we created a SERCA2b mutant (called Mut2b) containing the point mutation (N1035A), and then compared the Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+}-affinity to those of wild type SERCA2b and SERCA2a. We also performed coimmunoprecipitation to test for a protein-protein interaction between CRT and SERCA2b.

Ca\textsuperscript{2+}-ATPase activity was compared among wild type SERCA2a, SERCA2b, and Mut2b. As shown in Figure 8A, proteins were all expressed well. Note that the Mut2b protein showed the same tendency to aggregation as SERCA2b. This property, which is thought to be a consequence of the extended C-terminal sequence, is thus maintained when N1035 is changed to A. Enzyme activity was determined at 30°C using an enzyme-coupled spectrophotometric assay (see Experimental Procedures). The overexpressed enzyme activity was 10-20 fold higher than the endogenous SERCA activity of HEK-293T cells. The enzyme turnover rate was determined by normalizing the activity values to the level of expressed protein based on trapping of the catalytic intermediate. The endogenous SERCA activity was subtracted from each sample.

As shown in Figure 8B, wild type SERCA2a displayed ~1.6 fold higher turnover activity (1482±110 min\textsuperscript{-1}) compared to SERCA2b (860±52 min\textsuperscript{-1}), which was consistent with the results of others from COS cell microsomes (Lytton, et al., 1992; Verboomen, et al., 1994). The turnover of Mut2b (911±53 min\textsuperscript{-1}) was not significantly different from wild type SERCA2b.

C. Effect of point mutation N1035A on SERCA2b Ca\textsuperscript{2+} affinity

Calcium affinity is a critical parameter for the biological functioning of calcium pumps. It was investigated by monitoring the calcium dependence of Ca\textsuperscript{2+}-ATPase activity in the transfected HEK-293T cell system. Figure 9 shows that the apparent Ca\textsuperscript{2+} affinity of
FIG. 9. Ca\(^{2+}\) dependence of Ca\(^{2+}\)-ATPase activity. The Ca\(^{2+}\) dependence of the relative rate of Ca\(^{2+}\)-ATPase activity is shown for microsomes from HEK cells transfected with the SERCA2a (●), SERCA2b (■), and Mut2b (◆) constructs. The average from between 3 and 5 experiments are shown. The curves represent the best fit of the data to a cooperative model of enzyme activation obtained by non-linear curve-fitting. See “Experimental Procedures” for details. The derived \(K_{1/2}\) parameters ± the standard deviation for SERCA2a, SERCA2b and Mut2b, respectively, were: 0.23 ± 0.02 μM, 0.19 ± 0.01 μM, 0.18 ± 0.02 μM.
SERCA2b, with a $K_{0.5}$ of $0.19 \pm 0.01$ μM, was slightly higher than that of SERCA2a, with a $K_{0.5}$ of $0.23 \pm 0.02$ μM. The N1035A SERCA2b mutant showed a $K_{0.5}$ of $0.18 \pm 0.02$ μM, indistinguishable from that of SERCA2b. Despite the fact that these differences are sufficiently small to lack statistical significance, there is nevertheless a clear trend, with Mut2b resembling SERCA2b and not SERCA2a. Thus Mut2b retains the SERCA2b phenotype.

D. Co-immunoprecipitation

To determine whether CRT was physically associated with SERCA2b, coimmunoprecipitation was performed using 50 μg of microsomal protein isolated from HEK-293T cells transfected with either pcDNA3.1 empty vector or the human SERCA2b construct. The microsomes were solubilized in buffer containing either CHAPS or digitonin as the detergent, and in the presence or absence of Ca$^{2+}$. N1 anti-SERCA antibody was used for the precipitation, with pre-immune serum serving as the control. In pilot experiments, C4 antibody was also utilized. Immunoprecipitations with C4, however, were less efficient than those with N1 antibody. Immunoprecipitated proteins were resolved by 9% SDS-PAGE and then transferred to nitrocellulose. The blots were first probed for the presence CRT using C-terminal polyclonal chicken anti-CRT, and then stripped and blotted with monoclonal anti-SERCA IID8 antibody.

If the two proteins were associated, a band corresponding to the size of CRT should have been present on the gel. Figure 10 shows the results from a representative immunoprecipitation experiment. Six similar experiments were performed, all with similar results. Lanes with cell extract show SERCA expression in control, and much more expression in SERCA2b transfectants. The expression of endogenous CRT was also clearly evident. Interestingly, in this experiment, there was much less endogenous CRT in microsomes from SERCA2b overexpressing cells compared to the control microsomes.
FIG. 10. Co-immunoprecipitation of CRT with SERCA. Microsomes from HEK-293T cells, transfected with either pcDNA or SERCA2b constructs, were solubilized in detergent and immunoprecipitated (IP) with either anti-SERCA serum, N1 (panel B), or pre-immune serum (panel C). The precipitates were resolved by SDSPAGE, transferred to nitrocellulose, and probed with the anti-SERCA monoclonal antibody, IID8 (upper panel), or anti-CRT antibody, (lower panel). Panel A shows 2 μg of microsomes loaded on the gel directly. Microsomes were solubilized in either CHAPS (lanes 1 & 2) or in digitonin (lanes 3 & 4). The buffer contained either 1 mM EGTA (lanes 1 & 3) or 0.5 mM CaCl₂ (lanes 2 & 4).
However, this was not consistently observed with other microsome preparations. The blot with IID8 showed that SERCA was efficiently immunoprecipitated with the N1 antibody from both control and SERCA2b transfected microsomes (Figure 10B, upper panel), while no material was visible from immunoprecipitations with preimmune serum (Figure 10C). Note, however, that the combination of Ca\(^{2+}\) and digitonin dramatically reduced the efficiency of the immunoprecipitation. No associated CRT could be observed in any of the conditions used (Figure 10B & 10C, lower panels). Therefore, no CRT-SERCA2b interaction was found through the immunoprecipitation experiment. In an attempt to increase the immunoprecipitation efficiency, a pCDNA3.1(+)humanCRT construct was created and co-expressed with SERCA2b in HEK-293T cells. However, this approach was not successful due to the low expression level of recombinant CRT, such that transfected cells expressed almost the same amount as the endogenous CRT (data not shown).

In conclusion, the results described above demonstrated that the potential glycosylation site, N1035, was not critical for the functional difference between SERCA2a and SERCA2b in HEK-293T cells, nor did we observed any physical interaction between CRT and SERCA2b. Thus, the functional difference between SERCA2a and SERCA2b probably does not involve a lectin-dependent interaction with CRT. Therefore, our next strategy was to explore which portion of the SERCA2b C-terminal tail was needed for the functional difference between the two SERCA isoforms.

E. Characterization of SERCA2b deletion mutants

A previous publication has demonstrated that deletion of the last 12 amino acids in the SERCA2b C-termini is sufficient to convert the SERCA2b phenotype to a SERCA2a one (Verboomen, et al., 1994). To narrow down the critical subdomains further, we created a series of deletions from the C-terminal end, with each construct 3 amino acids
SERCA2a
NYLEP/AILE*997

SERCA2b
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTDTNFSDFMWS*1042

Mut2b
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTDTAFSDMFWS*1042

2b – 3
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTDTNFSDFMWS*1039

2b – 6
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTDTNFSDM*1036

2b – 9
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTD*1033

2b – 12
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVY*1030

2b + L
NYLEP/GKECVQPATKSCSFSACTDGISWPVFVLLIMPLVIWVYSTDTNFSDFMWS*1042

FIG. 11. C-terminal sequences of SERCA2a, SERCA2b and the SERCA2b mutants. The sequences downstream of amino acid 988 are shown in single letter code. The transmembrane domain is underlined. The point mutation N1035A (in the Mut2b construct), and the inserted L in the 2b+L construct, are shown in bold. The numbers indicate the position of the last amino acid of each construct.
shorter than the previous one. These constructs were called 2b-3, 2b-6, 2b-9, 2b-12, according to the length of the deletion (Figure 11). The upper panel of Figure 12A shows a representative immunoblot probed with monoclonal antibody C4, which illustrates the expression of SERCA2a, SERCA2b and SERCA2b mutants with deletions from the C-terminus of increasing length: 3, 6, 9, 12, as well as the insertion mutant. Both wild type and mutant pumps were expressed equally well. As previously described for SERCA2b, all the mutants showed a tendency to aggregation on the SDS-polyacrylamide gel. This indicates that the aggregation phenomenon is not due to the final 12 amino acids, and thus not correlated with the SERCA2b phenotype.

Pump turnover rate was compared among wild type SERCA2a, SERCA2b and the SERCA2b mutants. Mutant 2b-12, had an increased activity when compared to SERCA2b, that was similar to the activity of SERCA2a (Figure 12B). This result confirms the results from previous publications (Verboomen, et al., 1994). Surprisingly, all the other mutants, mutant2b-9, 2b-6, and 2b-3, also showed activity significantly different from SERCA2b and indistinguishable from SERCA2a. It was concluded from these experiments that the last 3 amino acids are of particular importance in determining functional difference between the two SERCA2 gene isoforms.

F. Effect of the orientation of the C-terminal tail on SERCA2b activity

To investigate whether the orientation or the length of the C-terminal tail might play a role in regulating SERCA2b activity, a leucine residue was inserted between the two leucines of the sequence VLLI in the 11th transmembrane domain of SERCA2b. This insertion was predicted to increase the length of the α-helix by one residue, thereby rotating the orientation of the C-terminal tail by ~100°. Since the inhibitory effect is only restricted to the last 3 amino acids of SERCA2b tail, the orientation of the tail might be critical for interactions involved in regulating SERCA2b activity. As shown in Figure 12B, the mutant
FIG. 12. Ca\textsuperscript{2+}-ATPase activity comparison. A, A representative immunoblot is shown. 2 μg of HEK-293T cell microsomes, transfected with the indicated SERCA constructs, were separated by SDS-PAGE, transferred to nitrocellulose, and probed with C4 anti-SERCA antibody. B, Ca\textsuperscript{2+}-ATPase activity comparison between SERCA2a, SERCA2b, the deletion constructs 2b-3, 2b-6, 2b-9, 2b-12, and the insertion construct 2b+L. Activity is the mean of the (turnover number/average turnover number for SERCA2b) ± the standard error of the mean for between four and nine determinations. Compared to SERCA2b, all other values were found to be significantly different, using a one-tailed paired t-test, with \( P \leq 0.01 \), except for 2b+L, which had \( P = 0.03 \).
2b+"L" also had significantly higher activity than SERCA2b, which indicated that the orientation of the tail was probably also involved in regulating SERCA2b activity.

**G. Peptide interaction with SERCA2a**

To investigate if the lower activity of SERCA2b compared to SERCA2a is due to the interaction of the last 12 amino acids with the Ca\(^{2+}\) transport domain of the protein, a peptide, NH\(_2\)-CSTDTNFSDMFWS-COOH, was synthesized that corresponds to the C-terminal 12 amino acids of SERCA2b plus an extra N-terminal cysteine residue. The peptide was dissolved in the same assay solution that we used to perform the activity measurement, with the pH adjusted to 8.0. We used canine cardiac sarcoplasmic reticulum (CSR), which expresses the SERCA2a protein, and compared the enzyme activity in the presence or absence of peptide. It was predicted that the peptide would interact with the luminal region of SERCA2a. To ensure access of the peptide to the luminal domain of the protein, CSR was solubilized for 40 min on ice (in 0.4 mg/ml C\(_{12}\)E\(_8\)) in the presence of 100 \(\mu\)M peptide, or in the buffer used to dissolve the peptide as a control. The concentration of the C\(_{12}\)E\(_8\) was optimized to maximize both the solubilization and the activity of the SERCA2a protein. The enzyme lost about 55% of its activity after solubilization. No significant change of enzyme activity was observed in the presence or absence of the peptide (Figure 13). To confirm that the activity that we were measuring was due to the SERCA protein, thapsigargin, which has been reported to inhibit SERCA activity specifically (Lytton, et al., 1991) was used. As shown in Figure 13, more than 80% of the solubilized ATPase activity was thapsigargin-inhibitable. Therefore, in this experiment, a possible inhibitory effect of the SERCA2b C-terminal peptide on SERCA2a activity was not observed.

It is possible that a closer association of tail with protein than could be achieved by the addition of soluble peptide would be needed to observe an inhibitory effect. Therefore,
FIG. 13. Influence of the synthetic SERCA2b C-terminal peptide on SERCA2a Ca^{2+}-ATPase activity. Cardiac sarcoplasmic reticulum (CSR) was used as a source of SERCA2a. CSR membranes were solubilized in the detergent C_{12}E_8, and activity was determined either in the presence (CSR+peptide) or absence (CSR+buffer) of 100 μM SERCA2b C-terminal peptide. The average ± standard error of the mean is shown for 3 measurements using the same CSR sample. The activity measured was inhibited ~80% by the addition of 200 nM thapsigargin (Tg).
an expression construct (denoted "2b entire tail") was prepared that encoded the last 49 amino acids of SERCA2b, which included the region between M10 and M11, M11 itself, and the luminal tail. A FLAG epitope was added to the N-terminus so that expression of the construct could be monitored. I planned to address the influence of this construct on SERCA2a activity, as well as investigate its interaction with SERCA through co-immunoprecipitation. Unfortunately, I was unable to perform these experiments since expression of the construct was not detected.
CHAPTER FOUR
DISCUSSION

A. Overview

The aim of this proposal was to understand the structure and mechanism that gives rise to the different properties of SERCA2a and SERCA2b. Structurally, SERCA2a and SERCA2b only differ at their C-termini, as described previously. The extreme C-termini of SERCA2a and SERCA2b are on opposite sides of the ER membrane, with the SERCA2b tail protruding in the ER lumen (Campbell, et al., 1992; Sachs, et al., 1995). Functionally, SERCA2b has a higher Ca\(^{2+}\) affinity, a two fold lower turnover rate, and a much lower vanadate-sensitivity than SERCA2a (Lytton, et al., 1992; Verboomen, et al., 1994). These properties are collectively referred to as the SERCA2b phenotype. The SERCA2b phenotype can be explained by a stabilization of the E\(_1\) conformation of the enzyme, thereby resulting in a slowing of the rate-limiting step of the catalytic cycle, E\(_1\)P(2Ca\(^{2+}\)) \(\rightarrow\) E\(_2\)P(2Ca\(^{2+}\)). Slowing of the E\(_1\)P \(\rightarrow\) E\(_2\)P transition will result in lower turnover. An increase in abundance of E\(_1\) (high Ca\(^{2+}\)-binding affinity, low vanadate affinity compared to E\(_2\)) at steady state will result in a higher apparent Ca\(^{2+}\) affinity and lower vanadate affinity.

Verboomen et al (1994) identified important subdomains in the SERCA2b tail by constructing SERCA2b mutants possessing deletions in this region of different lengths. It was concluded that the last 12 luminal amino acids of SERCA2b were of critical importance. Based on these results, I set out to identify more precisely the critical residues within the luminal C-terminal tail needed for the SERCA2b phenotype, and to explore possible mechanisms underlying this phenotype.
B. The influence of CRT for SERCA2b activity

CRT, an ER residential Ca\(^2+\) binding protein, has been reported to affect Ca\(^2+\) signaling in multiple cell systems (Treves, et al., 1990; Liu, et al., 1994; Bastianutto, et al., 1995; Coppolino, et al., 1997). One of its important functions is to modulate protein folding in the ER (Hammond, et al., 1994; Helenius, 1994; Nauseef, et al., 1995). It can specifically recognize and bind to the monoglycosylated form of immature glycoproteins in the ER lumen, ensuring that they achieve their correctly folded conformation (Ou, et al., 1993; Hammond, et al., 1994; Peterson, et al., 1995). In addition to this role, Camacho and her colleagues (John, et al., 1998) suggested that CRT modulates the function of SERCA2b through an interaction with an oligosaccharide chain linked to its potential glycosylation site at Asn1035.

The result of our experiments showed no alteration of SERCA2b activity by introducing the point mutation N1035A (Figure 8B and Figure 10), nor did co-immunoprecipitation show any interaction between CRT and SERCA2b in the presence or absence of CaCl\(_2\) (Figure 9). The outcome of our experiments seems, therefore, to be contradictory to Camacho’s recent publications (Camacho and Lechleiter, 1995; John, et al., 1998). These authors compared the properties of InsP\(_3\)-induced Ca\(^2+\) waves in Xenopus oocytes overexpressing different combinations of SERCA isoforms and CRT. They found that CRT only inhibited repetitive Ca\(^2+\) waves in SERCA2b-overexpressing oocytes but not in SERCA2a-overexpressing or control oocytes. They concluded that the SERCA2b-specific effect of CRT was dependent upon the presence of N1035 in the SERCA2b tail, and on the actions of the glucosidase inhibitor, deoxynojirimycin. As N1035 is a potential glycosylation acceptor site, these authors speculated that the functional influence of CRT on Ca\(^2+\) waves was mediated by a lectin-dependent association of CRT with SERCA2b, resulting in inhibition of SERCA2b activity.
There are several possible explanations for the disparity between our data and those of the Carnacho laboratory. First, Carnacho's data were observations in intact *Xenopus* oocytes, whereas our studies were done *in vitro* using microsomes isolated from HEK-293T cells. Possibly the functional effects of CRT on Ca\(^{2+}\) waves are very sensitive to small changes in SERCA2b activity that we are unable to detect under the conditions of our experiments. Factors or conditions necessary for a CRT-SERCA2b inhibitory interaction might be present in *Xenopus* oocytes but absent from HEK-293T cells. Alternatively, these factors or conditions might be present in whole cells but lost in microsomes. In this regard, it is important to bear in mind that the approach we chose to investigate the CRT-SERCA2b interaction (*co-immunoprecipitation*) requires a relatively stable interaction between two proteins, and weak interactions may result in the loss of association during the immunoprecipitation process (Kiselyov, et al., 1998). To optimize conditions for the *co-* immunoprecipitation experiment, HEK-293T cell microsomal proteins were solubilized in either 1\% CHAPS or 1\% digitonin. These detergents were chosen according to previous publications that reported successful immunoprecipitations between CRT and newly synthesized glycoproteins (Hebert, et al., 1996). To avoid losing possible associated CRT, the concentration of CHAPS was reduced to 0.3\% and the concentration of digitonin was reduced to 0.2\% in the washing steps. Furthermore, to investigate whether Ca\(^{2+}\) was required for a possible interaction with SERCA2b, precipitation was performed in either 0.5 mM CaCl\(_2\) or 1 mM EGTA, because the proline-rich P-domain of CRT has been reported to be involved in high affinity/low capacity Ca\(^{2+}\)-binding (\(K_d \approx 1 \mu M\), \(B_{\text{max}} = 1\) mol of Ca\(^{2+}\)/mole of protein), and bound Ca\(^{2+}\) has been found to be required for its lectin-like activity (Baksh and Michalak, 1991; Tjoelker, et al., 1994). Nevertheless, it is always possible that the conditions we employed were not optimized for some unknown parameters. Whatever the cause for the disparate observation between *Xenopus* oocytes and HEK-293T cells may be, we have to conclude that the difference in activity between
SERCA2a and SERCA2b observed in HEK-293T and COS cell microsomes cannot be due to a lectin-dependent association of CRT with SERCA2b.

Second, Camacho’s observation could be due, not to a direct inhibitory interaction of CRT on SERCA activity, but to a more indirect influence of CRT overexpression on either SERCA2b expression, or its localization in oocytes. No careful quantitative studies on SERCA expression in the presence or absence of CRT were reported in the studies of John, et al (1998). According to the findings of the Reithmeier laboratory (Popov, et al., 1997), using scanning N-glycosylation mutagenesis of extracellular loops of the anion exchanger, N-glycosylation sites had to be located more than 12 amino acids from the end of the preceding TMS and more than 14 amino acids away from the beginning to the following TMS to be glycosylated \textit{in vitro}. If their findings apply to the SERCA2b C-terminal tail, the location of N1035 seems to be too close to the end of M11 to be glycosylated (Figure 5). Note that the glycosylation status of N1035 has not been determined. If N1035 is not glycosylated, CRT would not be expected to interact with SERCA2b specifically.

C. Critical residues of the SERCA2b C-terminal tail underlying the functional difference between SERCA2a and SERCA2b

Through deletion mutagenesis, Casteel and colleagues (Verboomen, et al., 1994) previously demonstrated that the functional difference between SERCA2a and SERCA2b could be ascribed to the last 12 amino acids of SERCA2b. We constructed deletion mutants from the C-terminus of SERCA2b with increasing length: 3, 6, 9 and 12, to define further the functionally critical amino acids within these last 12 residues. As shown in Figure 12B, all the SERCA2b deletion mutants convert SERCA2b’s activity to SERCA2a, which suggests that the last 3 amino acids (FWS-COOH) are critical. The influence of the last 3 amino acids on the SERCA phenotype seems likely to be due to interactions with other
part(s) of the protein that result in stabilizing the Eᵢ conformation. In general, hydrophobic interactions have been suggested to be a major factor in protein-protein interactions (Chothia and Janin, 1975). Among the last 3 amino acids, two of them (FW) are hydrophobic and thus might contribute predominantly to the different phenotype of the two SERCA2 isoforms. It is also possible, however, that hydrogen bonding involving Serine, or ionic contacts involving the position of the negative carboxyl group at the C-terminus, are key to the SERCA2b phenotype. It will be interesting to mutate these amino acids in further investigations aimed at understanding the reason for reduced enzyme activity of SERCA2b compared to SERCA2a.

The role of the final 3 amino acids in the SERCA2b phenotype needs to be interpreted in light of two possible models which can explain the inhibitory effect of the luminal tail on SERCA2b activity (Figure 14). In model A, the luminal tail interacts directly with upstream luminal loops of SERCA2b, therefore stabilizing the Eᵢ conformation of the enzyme, and inhibiting the enzyme activity. This interaction is both necessary and sufficient to create the SERCA2b phenotype and may require not only the last 3 amino acids, but also a certain length or conformation of the tail. Based on model A, we predict that mutations in upstream luminal loops of SERCA2b should change SERCA2b activity; also, replacing M11 with different hydrophobic amino acids without changing the length or orientation of the luminal segment should have no effect on SERCA2b activity.

Model B is based on the model proposed for the inhibitory effect of PLN on SERCA2a: PLN modulates SERCA2a activity through transmembrane interactions in concert with cytoplasmic interactions. In model B, the interactions defining the SERCA2b phenotype are within the membrane, and the tail amino acids are required to bring M11 into the correct position with the other interacting transmembrane segments. Based on model B,
Fig. 14. Two possible models explaining the inhibitory mechanism of the SERCA2b C-terminal tail on SERCA2b activity. Model A, the last 3 amino acids as well as their orientation are required and sufficient to inhibit SERCA2b activity; Model B, the last 3 amino acids are required to allow another region of the tail (such as the transmembrane segment) to form inhibitory contacts.
we predict that replacing M11 with other amino acids without changing its length should result in changing SERCA2b activity. Also, coexpressing the “2b entire tail” construct (see Chapter 3, section F) with SERCA2a should result in inhibition of SERCA2a activity.

I have designed two experiments to test these two models. First, I tried to address the influence of the luminal tail on SERCA2a activity using a synthetic peptide, consisting of the last 12 amino acids plus an extra N-terminal cysteine residue. If the last 3 amino acids are necessary and sufficient for the inhibitory effect, SERCA2a was expected to convert to a SERCA2b phenotype by adding the peptide to the solubilized SERCA2a. The outcome of this experiment showed no change in SERCA2a activity in the presence or absence of the peptide, which is a support for model B. However, it is also possible that the experimental conditions were not sufficient to observe an inhibitory interaction. Second, the construct “2b entire tail” was created and coexpressed with SERCA2a. We expected to investigate the functional influence of the “2b entire tail” on SERCA2a activity, as well as the interaction of “2b entire tail”-SERCA2a by co-immunoprecipitation. However, I was unable to carry out these experiments since the construct “2b entire tail” was not expressed. This approach may still work if the construct can be altered to allow expression.

D. The significance of the orientation of the C-terminal tail

Since we predict that interactions between the C-terminal 3 amino acids and some other part(s) of the SERCA protein are essential for the SERCA2b phenotype, we also tested to see if the orientation of the luminal tail was important for the SERCA2b phenotype. By inserting an L between the two Ls of the sequence VLLI in the 11th transmembrane region, we anticipated that the length of the helix would be increased by around 1.5 Å and the orientation of the luminal tail rotated by ~ 100°. Depending upon how the hydrophobic segment sits in the membrane, this insertion may lengthen the tail as
well. This mutant showed increased SERCA2b activity, and a switch from SERCA2b to SERCA2a phenotype.

An explanation for this result is that the interaction of the SERCA2b luminal tail with upstream regions of the enzyme were weakened when the orientation of the tail was changed by 100°, or possibly by lengthening the luminal tail by one amino acid. Such a result suggests that the stability of the interaction requires a precise fit of the critical amino acids in the luminal tail to the more upstream part of the protein. The orientation or the length would be expected to be important for either model A or model B. In addition, however, for model B, where the inhibitory interaction resides in the transmembrane region, insertion of a leucine in the center of M11 may directly disrupt these interactions, therefore changing SERCA2b activity independently of any effect on the orientation or the length of the tail.

E. **Concluding remarks**

Our experiments have demonstrated that neither N1035 nor a putative oligosaccharide attached to that residue were necessary for the SERCA2b phenotype. The last 3 amino acids of the luminal tail of SERCA2b played the key role in underlying the functional difference between SERCA2a and SERCA2b. Adding an extra leucine residue to M11, which was expected to change the orientation of the tail, changed the SERCA2b phenotype. Future work will involve experiments designed to discriminate between model A and B, and further define critical residue(s) and conformation(s) of the tail that underlie the SERCA2b phenotype.
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