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The N Terminus of Sarcolipin Plays an Important Role in Uncoupling Sarco-endoplasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA) ATP Hydrolysis from Ca\(^{2+}\) Transport*

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Background: Both phospholamban (PLB) and sarcolipin (SLN) regulate SERCA activity, however, only SLN uncouples SERCA.

Results: The N and C termini of SLN, or the N terminus and transmembrane region of PLB, confer protein-specific function.

Conclusion: SLN N terminus plays a role in dynamic interaction and uncoupling of SERCA.

Significance: SERCA uncoupling by SLN increases heat production implicating SLN-SERCA interaction in muscle thermogenesis.

The sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is responsible for intracellular Ca\(^{2+}\) homeostasis. SERCA activity in muscle can be regulated by phospholamban (PLB), an affinity modulator, and sarcolipin (SLN), an uncoupler. Although PLB gets dislodged from Ca\(^{2+}\)-bound SERCA, SLN continues to bind SERCA throughout its kinetic cycle and promotes uncoupling of Ca\(^{2+}\) transport from ATP hydrolysis. To determine the structural regions of SLN that mediate uncoupling of SERCA, we employed mutagenesis and generated chimeras of PLB and SLN. In this study we demonstrate that deletion of SLN N-terminal residues 2ERSTQ leads to loss of the uncoupling function even though the truncated peptide can target and constitutively bind SERCA. Furthermore, molecular dynamics simulations of SLN and SERCA interaction showed a rearrangement of SERCA residues that is altered when the SLN N terminus is deleted. Interestingly, transfer of the PLB cytosolic domain to the SLN transmembrane (TM) and luminal tail causes the chimeric protein to lose SLN-like function. Further introduction of the PLB TM region into this chimera resulted in conversion to full PLB-like function. We also found that swapping PLB N and C termini with those from SLN caused the resulting chimera to acquire SLN-like function. Swapping the C terminus alone was not sufficient for this conversion. These results suggest that domains can be switched between SLN and PLB without losing the ability to regulate SERCA activity; however, the resulting chimeras acquire functions different from the parent molecules. Importantly, our studies highlight that the N terminus of SLN and PLB influence their respective unique functions.

The sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump is a P-type ATPase that catalyzes the transport of two Ca\(^{2+}\) ions into the lumen of the sarcoplasmic reticulum (SR), at the expense of energy from the hydrolysis of one ATP molecule. SERCA pump activity in cardiac and skeletal muscle is regulated by the Ca\(^{2+}\) ion gradient across the SR and by its regulatory proteins phospholamban (PLB) and sarcolipin (SLN), which physically interact with SERCA in a Ca\(^{2+}\)-sensitive manner. PLB is a 52-amino acid protein, consisting of a single transmembrane (TM) 22-residue α-helix connected to a 30-residue α-helical cytoplasmic domain. The role of PLB as an important regulator of cardiac contractility has been extensively studied. PLB modulates SERCA pump affinity for Ca\(^{2+}\) and binds to SERCA in the Ca\(^{2+}\)-free state. PLB phosphorylation has been shown to relieve inhibition of SERCA and accelerate Ca\(^{2+}\) uptake causing increased muscle relaxation. Compared with PLB, SLN is a much shorter 31-amino acid protein with a TM α-helix, an unstructured 7-residue cytosolic N terminus, as well as a unique C-terminal luminal tail that is highly conserved from mouse to human. Unlike PLB, SLN is expressed abundantly in skeletal muscle tissues but its expression in cardiac muscle is restricted to the atria, although it is reported to be up-regulated in the hypertrophied and failing ventricle.

Initial studies on SLN suggested that SLN is an uncoupler of the SERCA pump and could enhance heat production at the

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6 The abbreviations used are: SERCA, sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase; PLB, phospholamban; SLN, sarcolipin; TNP-ATP, trinitrophenyl adenosine monophosphate; TM, transmembrane; ANOVA, analysis of variance; TG, thapsigargin; aa, amino acid(s).
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expense of ATP hydrolysis (13, 14). Using SLN−/− and SLN overexpression mouse models, we recently showed that SLN is an important player in muscle thermogenesis and metabolism (4, 15–17). Our studies revealed that mice lacking SLN were unable to maintain body temperature when exposed to cold but could be rescued by re-expression of SLN. Additionally the SLN−/− mice developed obesity when fed a high fat diet suggesting SLN regulation of SERCA activity contributes to whole body energy metabolism (4). However, the mechanistic details of how SLN binding promotes uncoupling of SERCA resulting in heat production are unclear. Thus a major interest of our current research is to elucidate the molecular mechanism of the SLN-SERCA interaction.

Toward this goal we have developed a SLN and SERCA expression system in HEK cells and employed chemical cross-linking, SERCA ATP hydrolysis, and Ca2+ uptake assays to investigate how SLN interacts with SERCA during Ca2+ transport. Using this approach we recently showed that monomeric SLN binds to SERCA directly and the nature of SLN interaction with SERCA differs significantly from PLB (3, 4). A novel finding of this study was that although increasing the Ca2+ concentration reduced the interaction between SERCA and SLN, it did not abolish the interaction. Interestingly, SLN was also able to bind to SERCA in the Ca2+-bound, phosphoenzyme conformation of the pump, E1P. In contrast, PLB binds to SERCA only in the Ca2+-free E2 state of the pump (3, 8, 10, 18). These studies highlighted a key difference between SLN and PLB: that only SLN can bind to SERCA during Ca2+ transport and this unique property of SLN leads to uncoupling of SERCA. Despite these findings, the detailed mechanism of how SLN binding to SERCA promotes uncoupling of the pump and causes release of Ca2+ back to the cytosol is yet to be understood.

To date, attention has been focused on the C-terminal tail of SLN as the regulator of SERCA activity (19–21). Furthermore, recent x-ray crystallographic studies on co-crystals of SERCA-SLN and SERCA-PLB have added valuable information about the interaction of monomeric PLB or SLN with SERCA (22–24). However, these crystal structures were unable to identify where the N terminus of SLN or PLB interact with SERCA, due to the dynamic nature of the N terminus. Previous studies have found that the N-terminal domain of PLB is critical for the modulation of SERCA pump function (25–27). The most conspicuous structural differences between SLN and PLB lie in their N- and C-terminal regions, which may dictate their functional differences in SERCA regulation. Therefore, a major objective of this study was to investigate the structural regions of SLN necessary for interaction and uncoupling of SERCA. We especially investigated whether the N terminus regulates the uncoupling function of SLN. In the present study we generated chimeras by swapping SLN and PLB domains to test the specific function of each. Our studies for the first time demonstrate that the N terminus of SLN plays an important role in its uncoupling action on SERCA.

Experimental Procedures

Materials—Lipofectamine, DMEM, and other cell culture reagents were obtained from Invitrogen. The cross-linking reagent 1,6-bismaleimidohexane was purchased from Pierce (Thermo Scientific). Thapsigargin, sodium orthovanadate, AlCl3, and KOH were purchased from Sigma. 45CaCl2 was obtained from PerkinElmer Life Sciences.

Generation of SERCA, SLN, and PLB Constructs—The SLN, PLB, and SERCA constructs were generated by PCR amplification and cloning into pcDNA3.1(+) expression vector as reported previously (3). The rat SERCA1 cDNA sequence that has 99% homology with mouse SERCA1 was cloned into the pcDNA3.1(+) vector. The desired mutation of rat SERCA1, mouse SLN, and mouse PLB were done using the QuikChange™ site-directed mutagenesis kit (Agilent Technologies). All cDNA clones and mutated constructs were confirmed by direct sequencing. We have previously shown that E7C SLN and N30C PLB are similar in function to WT SLN and WT PLB (3). The mutants E7C SLN and N30C PLB were used in this study as native SLN and PLB, and also to generate all chimeras to enable cross-linking with SERCA at Cys318 (hence, E7C SLN and N30C PLB will be denoted as SLN and PLB throughout this text for simplicity, as shown in Fig. 1, A and B). Residues 2–6 of SLN were deleted to make the SLNNTdel mutant. The SLN N terminus (SLN residues 1–7) was replaced with the PLB N-terminal domain (PLB residues 1–30) to make Chimera-1. The last three residues (MLL)
in the C-terminal of PLB were replaced with SLN C-terminal tail residues, RSYQY to obtain Chimera-2. The length of PLB TM was kept intact to prevent possible mis-localization of PLB (28), as well as to ensure ideal interaction of RSYQY with SERCA. The N-terminal residues 1–30 of Chimera-2 were replaced with residues 1–7 of SLN to get Chimera-3 (Fig. 1, A and B). We further mutated residues 3RS to AA, 5TQ to AA, as well as 3RSTQ to AAAA to obtain N-terminal alanine mutants of SLN. Protein expression of the chimeras was determined using the SLN antibody, which detects the C-terminal RSYQY sequence of SLN. PLB expression was determined by PLB antibody, which recognizes residues 2–13 of PLB N-terminal domain.

Expression of Proteins and Microsome Preparation—HEK293 cells were co-transfected with SERCA and SLN or PLB cDNAs at a 1:2 ratio, using Lipofectamine 2000. The cells were harvested in PBS 48 h post-transfection and the pellet was flash frozen in liquid nitrogen and stored at −80°C. Microsomes from transfected cells were prepared as described previously (3).

SERCA-mediated Ca2+ Uptake and ATP Hydrolysis Assays—Oxalate-supported Ca2+ uptake assay was performed to determine the effect of SLN, PLB, or their mutants on SERCA-mediated Ca2+ transport. The proteins were expressed in HEK cells by transfection of DNA using Lipofectamine (3). HEK homogenates were incubated in Ca2+ uptake buffer with the desired free Ca2+ level as determined by Maxchelator webeware. Aliquots of the reaction were collected and filtered at 30, 60, and 90 s. The filters were washed and bound radioactive 86Ca2+ was measured (29). ATP hydrolysis activity was measured using the Biomol green phosphate assay reagent (3, 30). The reaction was initiated by addition of 5 μg of microsomes in the Ca2+ uptake buffer and samples were collected at the end of 30 min. The amount of P, release was calculated as nanomole of P/mg/min. Values of reactions carried out in the presence of thapsigargin (TG), a SERCA inhibitor, were subtracted from all samples to obtain SERCA specific activity (3).

All ATP hydrolysis and Ca2+ uptake assays were carried out with a SERCA control for each sample. The SERCA protein level in the samples was normalized to that in the control to obtain the final absolute activity values. The final values were expressed as a percentage of the maximum control SERCA activity. Activity curves with n = at least 3 measurements were generated using sigmoidal dose-response curve fitting in the GraphPad Prism 6 software to get the best-fit values ± S.E. Analysis of data were done using ANOVA followed by Dunnett’s multiple comparisons test.

Chemical Cross-linking—Chemical cross-linking of proteins was performed with the 10-Å homo-bifunctional sulphydryl cross-linker, 1,6-bismaleimidohexane, as previously described (3). Briefly, 15 μg of microsomes were mixed with 3 mM ATP in cross-linking buffer followed by addition of 0.1 mM cross-linker. The reaction was stopped after incubation for 1 h at 25°C, by the addition of SDS-PAGE sample-loading buffer containing 100 mM dithiothreitol. Specific cross-linking of SLN, SLNNTdel, Chimera-1, and Chimera-3 to SERCA showed a band above 110 kDa probed with anti-SLN antibody, whereas PLB or the Chimera-2 interaction with SERCA showed a 116-kDa band probed with anti-PLB antibody. The effect of TG was studied by adding 0–10 μM TG to the reaction mixture without Ca2+, in the presence of ATP, before the addition of cross-linker (3).

Cross-linking to Different Kinetic States of SERCA—Stable analogs of the major intermediates of SERCA kinetic steps (E2, E1, E1Pca2+-ADP, and E2P) were obtained by incubating microsomes in a cross-linking reaction buffer for 45 min at 25°C with different chemicals, before addition of 1,6-bismaleimido-hexane (3, 31, 32). Incubating the microsomes without ATP induced the E2 state. 100 μM free Ca2+ with 3 mM ATP in the buffer resulted in a prevalence of the E1-PcA2 state. Incubation with 50 μM AlCl3 and 3 mMKF and 3 mM ADP resulted in formation of the E1-AlF4-ADP complex, an E1PcA2-ADP analog. Last, the analog of the E2P to E2 transition state (E2-AlF4-) was produced by addition of 50 μM AlCl3 and 3 mMKF to the buffer.

MD Simulations—The simulations were performed with the GROMACS 5.0 program (33). For modeling the system we used the crystal structure of SERCA and SLN in the Mg2+-bound E1 state (Protein Data Bank code 3W5A) (24). Here the ATP analog TNP-AMP was deleted, whereas the bound Mg2+-ion and crystal waters were retained. For the truncated version we deleted residues 2–6 of SLN after equilibration. We used the same protonation scheme as Espinoza-Fonseca et al. (34), namely that, Ca2+-binding residues Glu771, Asp1006, and Glu309 were kept unprotonated, whereas Glu908 was protonated. The system consisted of SERCA, SLN, 500 POPC molecules, and ~55,000 TIPS3P water molecules. The protein was inserted into a pre-equilibrated bilayer using the “membed” option of GROMACS 5.0 (35). Furthermore, Na+ and Cl− ions were added to ensure a salt concentration of 150 mM. Virtual sites were used for all hydrogens in the system (36) allowing for a 5-fs time step in conjunction with the CHARMM36 force field (37). Both systems were run for 200 ns of which 50 ns were discarded as equilibration. Periodic boundary conditions were used, electrostatic forces were calculated using the Particle Mesh Ewald (38) method with a real space cut off of 1.2 nm, and the non-bonded interactions were calculated using a force switch in the range 1.0 to 1.2 nm. The temperature was kept at 310 K using the Nose-Hoover thermostat (39, 40) with a 10-ps time constant, and the pressure was coupled semi-isotropically using the Parrinello-Rahman barostat (41) as required by the virtual sites method, a reference value of 1.0 bar and a time constant of 50 ps. All bonds were constrained with LINCS (42).

Results
Deletion of SLN Cytosolic Portion (N-terminal 2–6 Residues) Leads to Loss of SLN Uncoupling Effect on SERCA—SLN has a unique unstructured N terminus consisting of 7 aa, whereas PLB has a 30-aa long α-helical N-terminal domain. X-ray crystallographic studies on SLN-SERCA co-crystals were unable to map the location of the N terminus and the interaction of N-terminal residues with SERCA remains a mystery so far (22, 24). We therefore wanted to determine whether the unique N-terminal residues are responsible for the uncoupling action of SLN on SERCA. Toward this goal, we first deleted the N-terminal 2–6 residues from SLN (SLNNTdel). The deleted con-
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**FIGURE 2. Deletion of the cytosolic portion (residues 2–6) of SLN results in loss of function.** A, SERCA was expressed with SLN or SLN<sup>NTdel</sup> in HEK cells. Cross-linking was done in the presence of different concentrations of Ca<sup>2+</sup>. Representative immunoblot shows Ca<sup>2+</sup>-dependent cross-linking of SLN and SLN<sup>NTdel</sup> with SERCA1. Protein band intensities were measured and plotted as the percentage of band intensity of reaction without Ca<sup>2+</sup> (n = 3–4 measurements) (right panel). B, SERCA ATP hydrolysis activity was measured in reactions containing SERCA alone and SERCA with SLN or SLN<sup>NTdel</sup>. The values are given as the percentage of SERCA V<sub>max</sub> (n = 6 measurements) (left panel). K<sub>0.5</sub> (pCa) values of SERCA affinity for Ca<sup>2+</sup> were calculated and compared with control SERCA (right panel). C, SERCA calcium uptake was determined in reactions containing SERCA alone and SERCA with SLN or SLN<sup>NTdel</sup>. The values are given as mean ± S.E. ** or *** = SLN V<sub>max</sub> is reduced as compared with SERCA V<sub>max</sub> (p < 0.01 as analyzed by ANOVA).

struct was co-expressed with SERCA in HEK cells. The mutant protein was expressed at high levels and localized to the ER. Microsomes containing SERCA and SLN<sup>NTdel</sup> were then cross-linked at increasing Ca<sup>2+</sup> concentrations. As shown in Fig. 2A, SLN interacted with SERCA at all Ca<sup>2+</sup> concentrations tested, however, cross-linking intensity was reduced with increasing Ca<sup>2+</sup> concentration (3, 4). SLN<sup>NTdel</sup> was also able to cross-link with SERCA; however, the intensity of the cross-linking remained unchanged with increasing Ca<sup>2+</sup> concentration (Fig. 2A). This data suggested that SLN<sup>NTdel</sup> lost its ability to interact with SERCA in a Ca<sup>2+</sup>-sensitive manner but it remained bound to SERCA constitutively. We further tested if SLN<sup>NTdel</sup> affected SERCA activity by performing SERCA ATP hydrolysis and Ca<sup>2+</sup> uptake assays. Our results showed that both SLN and SLN<sup>NTdel</sup> had no significant effect on ATP hydrolysis activity (Fig. 2B). As expected, SLN decreased the V<sub>max</sub> of Ca<sup>2+</sup> uptake by 37% (Fig. 2C), which is in agreement with our previous study (3). Interestingly SLN<sup>NTdel</sup> had no effect on SERCA Ca<sup>2+</sup> uptake (Fig. 2C). These data suggested that although SLN<sup>NTdel</sup> was able to bind to SERCA constitutively, the deletion of the SLN N terminus made the molecule functionally inactive and it no longer affected SERCA ATP hydrolysis activity or Ca<sup>2+</sup> uptake.

Replacing SLN N Terminus (1–7 aa) with PLB N-terminal Domain (1–30 aa) Confers PLB-like Characteristics—The above studies pointed out that deletion of the SLN N terminus resulted in a loss of function and clearly suggested that the N terminus is an important functional region. We then studied if the SLN function could be restored by addition of a non-homologous sequence to the N terminus. For this, we added the FLAG tag (DYKDDDDK) onto the N terminus of SLN<sup>NTdel</sup>. Similar to SLN<sup>NTdel</sup>, the resulting peptide was able to bind to SERCA at all Ca<sup>2+</sup> concentrations tested but had no effect on SERCA function (data not shown) and behaved similar to SLN<sup>NTdel</sup>. We next wanted to determine whether adding the PLB cytosolic domain (1–30 aa) (that has been shown to be important for PLB regulation of SERCA) to the N-terminal-truncated SLN would confer PLB-like function to the resulting Chimera-1 (Fig. 1B). We therefore compared the SERCA-Chimera-1 interaction with that of SERCA-SLN as well as SERCA-PLB at increasing Ca<sup>2+</sup> concentrations. As shown in Fig. 3A, SLN and PLB behaved as we have earlier reported (3); i.e., SLN was able to interact with SERCA at all Ca<sup>2+</sup> concentrations tested but PLB interaction with SERCA was abolished at higher Ca<sup>2+</sup> concentrations. Interestingly, Chimera-1 interacted with SERCA up to 1 μM and the cross-linking was abolished at 2 μM Ca<sup>2+</sup> (Fig. 3A), thus Chimera-1 behaved like PLB. The ATP hydrolysis assay showed that like PLB, Chimera-1 decreased the K<sub>0.5</sub> of SERCA activity (K<sub>0.5</sub> SERCA = pCa 6.787 ± 0.039; K<sub>0.5</sub> SERCA + Chimera-1 = pCa 6.638 ± 0.014, p < 0.05; K<sub>0.5</sub> SERCA + PLB = pCa 6.248 ± 0.063, p < 0.0001, mean ± S.E.) (Fig. 3B). The Ca<sup>2+</sup> uptake assay showed that Chimera-1 did not inhibit the V<sub>max</sub> or affinity of SERCA for Ca<sup>2+</sup> (K<sub>0.5</sub> SERCA = pCa 6.745 ± 0.02; K<sub>0.5</sub> SERCA + Chimera-1 = pCa 6.535 ± 0.134; K<sub>0.5</sub> SERCA + PLB = pCa 6.358 ± 0.078, p < 0.01, mean ± S.E.) (Fig. 3C). These results collectively suggest that replacing the SLN N terminus with the PLB N terminus leads to a loss of SLN-like function in the resulting Chimera-1, despite the fact that it has both the TM and C-terminal residues of SLN.

**SLN N Terminus Plays a Role in Uncoupling the SERCA Pump**—We additionally generated Chimeras-2 and -3 to determine the functional relevance of the SLN C and N terminus. In Chimera-2, the C-terminal residues of PLB (MLL) were replaced with the C-terminal residues of SLN (RSYQY). To test the role of the SLN N terminus we created Chimera-3, using Chimera-2 from which the N terminus of PLB was replaced with that of SLN (MERSTQ) (although this chimera contained the TM domain of PLB, no PLB antibody specific for this region is available). We therefore had to replace the C-terminal residues of PLB (MLL) with those of SLN (RSYQY) for immunodetection.) SLN, PLB, and the chimeras were each expressed with SERCA in HEK cells and cross-linking and functional assays were carried out as described above. Cross-linking studies showed that the PLB interaction with SERCA was highly sensitive to Ca<sup>2+</sup> and was abolished between 1 and 2 μM Ca<sup>2+</sup>. On
the other hand, Chimera-2 interaction with SERCA was stronger and persisted even at 2 μM Ca²⁺ but was abolished at 5 μM Ca²⁺ (Fig. 4A), suggesting that the addition of the SLN C terminus (RSQY) residues onto PLB increased its affinity to SERCA. Interestingly, Chimera-3 cross-linked to SERCA at all minus (RSQY) residues onto PLB and increased its affinity to SERCA in HELK cells. Cross-linking was done in the presence of different concentrations of Ca²⁺. The representative immunoblot shows Ca²⁺ induced alteration in cross-linking of PLB, Chimera-2, and Chimera-3 with SERCA. Protein band intensities were measured and plotted as the percentage of band intensity of reaction without Ca²⁺ (n = 3–4 measurements) (right panel). B, SERCA ATP hydrolysis activity was measured in reactions containing SERCA alone and SERCA with PLB or Chimera-1 at various Ca²⁺ concentrations. The values are given as percentage of SERCA Vₘₐₓ (n = 3–6 measurements) (left panel). Kₐ₅ (pCa) values of SERCA affinity for Ca²⁺ were calculated and compared with control SERCA (right panel). C, Ca²⁺ uptake was determined in reactions containing SERCA alone and SERCA with PLB or Chimera-1. The values are given as the percentage of SERCA Vₘₐₓ (n = 4 measurements) (left panel). Kₐ₅ (pCa) values of SERCA affinity for Ca²⁺ were calculated and compared with control SERCA (right panel). * = p < 0.05; ** = p < 0.01; **** = p < 0.0001, as analyzed by ANOVA. Data are mean ± S.E.

To further determine how Chimeras-2 and -3 affect SERCA activity, we measured SERCA ATP hydrolysis and Ca²⁺ uptake. The ATP hydrolysis assay showed that similar to PLB, the presence of Chimera-2 decreased the affinity of the SERCA pump for Ca²⁺, without a significant effect on Vₘₐₓ of the pump (Kₐ₅ SERCA = pCa 6.595 ± 0.044; Kₐ₅ SERCA + Chimera-2 = pCa 6.378 ± 0.05, p < 0.01, mean ± S.E.). The Ca²⁺ uptake assay also showed that Chimera-2 decreased the apparent affinity of SERCA for Ca²⁺ uptake, without affecting Vₘₐₓ (Kₐ₅ SERCA = pCa 6.785 ± 0.023; Kₐ₅ SERCA + Chimera-2 = pCa 6.398 ± 0.065, p < 0.0001; mean ± S.E.), suggesting that Chimera-2 retains PLB-like function. These results revealed that although the C terminus of SLN increases the affinity of Chimera-2 to SERCA, it functions like PLB. When we swapped the N terminus of Chimera-2 with that of SLN, the resulting Chimera-3 did not affect ATP hydrolysis activity (Kₐ₅ SERCA = pCa 6.595 ± 0.044; Kₐ₅ SERCA + Chimera-3 = pCa 6.609 ± 0.044, mean ± S.E.) (Fig. 4B) and behaved similar to SLN. The Ca²⁺ uptake assay showed that Chimera-3 did not affect the apparent affinity of SERCA for Ca²⁺ uptake (Kₐ₅ SERCA = pCa 6.785 ± 0.023; Kₐ₅ SERCA + Chimera-3 = pCa 6.706 ± 0.058, mean ± S.E.) but showed a 25% decrease in the Vₘₐₓ of SERCA pump (Fig. 4C). These data using Chimera-3 suggest that replacing the PLB N terminus of Chimera-2 with that of SLN converts it to a SLN-like molecule.

The N Terminus of SLN Enables Its Dynamic Interaction with SERCA—The SERCA pump undergoes four major kinetic steps (E2, E1P, E2P, and E2) during Ca²⁺ transport (1). We have
previously shown that SLN interacts with SERCA through various kinetic steps of the Ca\(^{2+}\) transport cycle, whereas PLB cannot bind to Ca\(^{2+}\)-bound SERCA (3). Therefore we investigated if SLN binding to SERCA in its various kinetic states is dependent on the N terminus of SLN, by the use of these chimeras. We assessed the ability of each chimera to bind to the five different kinetics states of SERCA as described previously (3). We measured protein cross-linking in the E2 state with and without ATP; the Ca\(^{2+}\)-bound phospho-intermediate and transition state, as well as the E2P state. Our cross-linking results showed that only SLN and Chimera-3 having the SLN N terminus were able to interact with SERCA across all tested kinetic states. However, PLB, Chimera-1, and Chimera-2 (with the PLB N terminus) bound only to the Ca\(^{2+}\)-free E2 conformation and this interaction was enhanced in the presence of ATP (Fig. 5A).

It has been previously shown that PLB cannot interact with Ca\(^{2+}\) bound SERCA, whereas SLN can (3, 4). To further determine how Ca\(^{2+}\) binding to both sites in SERCA affect its interaction with the different chimeras, we performed cross-linking with the D351A-SERCA mutant. The D351A-SERCA mutant can bind two Ca\(^{2+}\) ions but cannot hydrolyze ATP; therefore in the presence of Ca\(^{2+}\) and ATP at pH 7.0, all the pumps remain in a Ca\(^{2+}\)-bound state. Our results showed that SLN and Chimera-3 (having SLN N terminus) were able to bind to D351A-SERCA but Chimeras-1 and -2 (with the PLB N terminus) bound only to the Ca\(^{2+}\)-free E2 conformation and this interaction was enhanced in the presence of ATP (Fig. 5A).

Ala

Alanine Mutagenesis of SLN Cytosolic Residues Alters Ca\(^{2+}\)-dependent Interaction with SERCA

The N terminus of SLN (MERSTQ) is relatively short and the ability of SLN to remain bound to SERCA may involve specific interactions of the N terminus with SERCA. To test this idea we mutated the N-terminal residues to alanines and analyzed the ability of these mutants to bind to SERCA in a Ca\(^{2+}\)-dependent manner. We generated double alanine mutants at positions 3, 4 (3RS-AA), and 5, 6 (5TQ-AA) at the N terminus of SLN. In addition we mutated all 4 residues together to alanine (3RSTQ-AAAA) to determine the effect of these residues on the function of SLN. Our chemical cross-linking results showed that the 3RS-AA mutant had a minimal effect on SLN cross-linking with SERCA at a high Ca\(^{2+}\) concentration. The 5TQ-AA mutant showed decreased interaction with SERCA at a high Ca\(^{2+}\) concentration, whereas mutation of all 4 residues resulted in further reduction of the interaction, which was abolished at 2 \(\mu\)M Ca\(^{2+}\) (Fig. 6). We also performed SERCA functional assays in the presence of these SLN mutants to study the role of the N-terminal residues (Table 1). Although the \(V_{\text{max}}\) values were low in the presence of the mutant SLNs, we found no significant difference in the ATP hydrolysis activity of SERCA when expressed with the mutants, as compared with SERCA alone. Additionally we observed that only the 3RSTQ-AAAA SLN mutant significantly reduced the \(V_{\text{max}}\) of SERCA Ca\(^{2+}\) uptake, similar to native SLN. Although the SLN 3RS-AA and 5TQ-AA lowered the \(V_{\text{max}}\) of SERCA Ca\(^{2+}\) uptake, this reduction was not significantly different from the SERCA + SLN \(V_{\text{max}}\)

Uncoupling of SERCA by Sarcolipin

SLN, PLB, and their chimeras were cross-linked with different SERCA kinetic steps induced by chemical analogs as described previously (3). B, SLN, PLB, and their chimeras were expressed with D351A-SERCA, and proteins were cross-linked at different Ca\(^{2+}\) concentrations. C, SLN\(^{NT\text{del}}\) Chimeras-1, -2, and -3 were cross-linked with SERCA in the presence of ATP and increasing concentrations of TG. Cross-linked proteins were immunoblotted with SLN or PLB antibody.

**FIGURE 5.** Chimeric proteins can dynamically interact with SERCA. A, SLN, PLB, and their chimeras were cross-linked with different SERCA kinetic steps induced by chemical analogs as described previously (3). B, SLN, PLB, and their chimeras were expressed with D351A-SERCA, and proteins were cross-linked at different Ca\(^{2+}\) concentrations. C, SLN\(^{NT\text{del}}\) Chimeras-1, -2, and -3 were cross-linked with SERCA in the presence of ATP and increasing concentrations of TG. Cross-linked proteins were immunoblotted with SLN or PLB antibody.
The SLN N Terminus Interacts with Cytosolic Residues of SERCA—To investigate the interactions of the N terminus of SLN with SERCA; we performed molecular dynamics (MD) simulations of a SERCA/POPC system with an intact SLN-bound SERCA and with the N-terminal truncated SLN (SLNNTdel) bound SERCA. We found several changes in SERCA residues both at and surrounding the site of interaction with the SLN N terminus.

The MD simulations showed a salt bridge between Arg3 of SLN and Glu45 of SERCA. Glu45 is located on the loop connecting the cytosolic domain and M1 as shown in Fig. 7A. In SERCA + SLN, Glu45 is localized near, and is oriented toward, the N terminus of SLN, whereas in SERCA + SLNNTdel, this interaction is lost and thus Glu45 fluctuates and is in general, oriented toward the bulk. The removal of the N terminus causes Glu45 to have different interaction patterns with residues Trp107, Arg110, Asn111, and Asn114 in the cytosolic region of the SERCA M2 helix in the two simulations as shown in Fig. 7B. In SERCA + SLNNTdel, all the interactions between Glu45 and SERCA residues near the N terminus of SLN are lost, consistent with the change in spatial and orientational behavior.

The simulations also showed that residue Glu2 of SLN forms a salt bridge with Arg324 of SERCA, which is located on the cytosolic half of the M4 helix (M4C) and is oriented toward the groove in SERCA where SLN is bound. This helix M4C has been previously implicated in inter-domain communication in the SERCA Ca2+/H11001 transport cycle (43, 44). Fig. 7C shows representative snapshots from the two systems. In SERCA + SLNNTdel, the loss of this salt bridge results in changes in interactions with Asn111, Asn114, and Ala118.

<table>
<thead>
<tr>
<th>TABLE 1 The effect of SLN N-terminal alanine mutants on SERCA ATP hydrolysis activity and calcium uptake</th>
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<tr>
<td>The Kₜₒ₅ values are in pCa units. The Vₖₒ₅ values are expressed as a percentage of control SERCA Vₖₒ₅. Results are mean ± S.E. for n = 3 measurements.</td>
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<th></th>
<th>ATP hydrolysis activity</th>
<th>Calcium uptake</th>
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<tr>
<td></td>
<td>Kₜₒ₅</td>
<td>Vₖₒ₅</td>
</tr>
<tr>
<td>SERCA</td>
<td>6.52 ± 0.06</td>
<td>97.97 ± 1.18</td>
</tr>
<tr>
<td>SERCA + SLN</td>
<td>6.57 ± 0.13</td>
<td>97.18 ± 11.27</td>
</tr>
<tr>
<td>SERCA + SLN₅RS-AA</td>
<td>6.61 ± 0.04</td>
<td>87.47 ± 8.03</td>
</tr>
<tr>
<td>SERCA + SLN₅TQ-AA</td>
<td>6.61 ± 0.06</td>
<td>84.86 ± 6.83</td>
</tr>
<tr>
<td>SERCA + SLN₅RSTQ-AAAA</td>
<td>6.60 ± 0.04</td>
<td>80.46 ± 5.00</td>
</tr>
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</table>

* p < 0.05, as compared to control SERCA values, analyzed by ANOVA.
Discussion

A major objective of this study was to determine the structural regions that enable SLN interaction with SERCA and promote uncoupling of the pump from Ca\(^{2+}\) transport. We recently showed that monomeric SLN binds to the TM groove on SERCA consisting of TMs M2, M6, and M9, a region previously characterized to bind PLB (45). The binding of SLN to this groove, however, involves a different set of SERCA residues (3, 45). Most importantly, we showed that SLN remains bound to SERCA in the presence of high Ca\(^{2+}\), whereas PLB is displaced from the groove. We also showed that SLN interacts with SERCA throughout the Ca\(^{2+}\) transport cycle and proposed that its ability to interact with SERCA during the Ca\(^{2+}\) transport cycle promotes uncoupling of the SERCA pump. These and other published studies suggest that PLB is an affinity modulator of SERCA (inhibits SERCA at submaximal Ca\(^{2+}\) concentrations), whereas SLN is an uncoupler of the pump and decreases \(V_{\text{max}}\) of Ca\(^{2+}\) uptake (3, 7, 10, 13, 46, 47). It is, however, unclear how SLN is able to uncouple the SERCA pump.

To date, the structural components that enable SLN to uncouple SERCA remain to be characterized. SLN is a short 31-amino acid protein consisting of an unstructured 7-residue (MERSTQE) N terminus, a TM α-helix made of 19 residues, and a unique 5-residue (RSYQQ) C-terminal luminal tail (48–51). Previous functional studies on SLN suggested that the C-terminal RSYQQ sequence is important for SERCA regulation (19–21). The published x-ray crystallographic studies by Toyoshima et al. and Winther, Bublitz, and co-workers (22, 24) are an important milestone, which has shown that SLN indeed binds to the M2, M6, and M9 TM groove and stabilizes the E1 state of SERCA. The crystal structures showed interaction in the TM region but were unable to identify the N terminus locations of SLN on SERCA; therefore the nature of the SLN N terminus interaction with SERCA and its role in regulating SERCA function have remained elusive. We knew that SLN and PLB bind to the same SERCA groove but produce different functional outcomes (3, 13–14, 45). Hence, we chose to generate chimeras between PLB and SLN to dissect their unique functional domains and determine if the domains could be exchanged without losing function. Results from our study show that we were successful in creating functional chimeras. Moreover, these chimeras inherited the function of their respective N terminus.

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The TM and C Terminus Are Sufficient for SLN Localization and Interaction with SERCA—Despite recent progress in localizing SLN to the SERCA TM groove, it remained unclear if the uncoupling function could be localized to a particular portion or a set of residues in SLN. In the present study, we focused our
attention on investigating the role of the SLN N terminus in the uncoupling of SERCA. We found that deletion of the N terminus (ERSTQ) of SLN disabled its function (Fig. 2). The mutant peptide (SLN$^{NTdel}$) constitutively bound to SERCA, failed to show Ca$^{2+}$ sensitivity, and had no effect on SERCA Ca$^{2+}$ transport. We have earlier shown that native SLN or PLB are displaced from SERCA by TG, a SERCA inhibitor, which locks the pump irreversibly in the E2 conformation (3). Interestingly, the SLN$^{NTdel}$ peptide could not be dislodged from SERCA in the presence of TG (Fig. 5C). Thus SLN without its N terminus remained in the TM groove and lost its ability to dynamically interact with SERCA. These studies suggest that the occupation of the SERCA groove by the truncated SLN peptide is not sufficient to cause uncoupling. At the same time this mutant could successfully localize to SERCA, which suggests that the TM and C terminus are vital for SLN localization and interaction with SERCA in the TM groove (21), which is also in agreement with recent x-ray crystallographic studies (22, 24).

The N Termini of SLN and PLB Are Essential for Their Unique Function—Because the truncated SLN peptide failed to show any effect on SERCA Ca$^{2+}$ transport, we added a heterologous N-terminal FLAG tag and also the N-terminal domain (1–30 aa) of PLB (Chimera-1) to observe an effect on SERCA. Only the addition of the PLB N terminus showed an effect on SERCA function and caused Chimera-1 to decrease the apparent affinity of SERCA but not $V_{\text{max}}$ (Fig. 3). This is in agreement with previous findings showing that the inhibitory function of PLB can be localized to the N terminus (25, 26, 52).

Similarly, the PLB N terminus, not the SLN C terminus, determined the function of Chimera-2. Although the SLN C terminus increased the binding affinity of Chimera-2 to SERCA, which is in agreement with recent studies by Gorski et al. (20), we, however, found that it was unable to promote SERCA uncoupling in the presence of the PLB N-terminal domain (Fig. 4). An interesting observation was that Chimera-2 behaved more like PLB than Chimera-1. The only variable factor between these two chimeras is the TM segment, which suggests that the TM of PLB plays a significant role in defining PLB function. Previous findings showing that the inhibitory function of PLB can be switched easily without affecting PLB properties. These studies employing chimeras allowed us to conclude that the N termini of SLN and PLB influence their respective identity as an uncoupler or affinity modulator. However, the TM and C terminus are important for SLN localization within the SERCA groove and without them SLN will not be able to interact with SERCA. In this study we additionally show that the TMs of PLB and SLN could be switched without compromising SLN function.

Although the above studies identified the SLN N terminus as an important uncoupling portion, it remained unclear how this region interacts with SERCA in a Ca$^{2+}$-sensitive manner. Therefore we mutagenized the N-terminal RSTQ residues into alanines and found that mutagenesis from 2 to 4 residues increasingly weakened the mutants’ ability to bind to SERCA (Fig. 6) as compared with native SLN. This could be a result of unfavorable interaction between SERCA residues and the N-terminal alanines on SLN, causing a change in the orientation of the SLN TM and C terminus within the SERCA binding groove. On the other hand, compared with alanine mutagenesis, deletion of the N terminus caused the truncated SLN peptide (SLN$^{NTdel}$) to constitutively bind to SERCA even at a high 5 $\mu$M Ca$^{2+}$ concentration and unlike native SLN, it lost its SERCA regulating function and remained bound in the presence of TG. The SLN N terminus is thus important for its binding and dynamic interaction with SERCA during the kinetic cycle of the pump. The N-terminal alanine mutations, however, did not significantly alter the functional effect of SLN on SERCA (Table 1). It should be noted that these mutagenesis studies are only preliminary and further investigation with exhaustive mutagenesis using a range of amino acids is necessary to provide a definitive picture of how the side chain interactions in this region affect SLN function.

Evolution and Functional Divergence of SLN and PLB—Our studies using native SLN, PLB, and their chimeras, show that domain function can be separated and the domains can function independently of each other. The very fact that they both bind to the same SERCA groove, and that the two proteins have retained considerable homology in the TM region, provide support to their common origin. Recent studies in Drosophila have identified an ancestral protein, Sarcolamban, as a regulator of SERCA, which shares most homology in the TM region with PLB and SLN. This finding and studies on the SLN and PLB structure suggest that these proteins must have evolved from a common ancestral gene involved in Ca$^{2+}$ homeostasis (53). However, somewhere during evolution, the ancestral gene duplicated and diverged to code for PLB and SLN with very distinct functions even before the dawn of vertebrates (53, 54). Thus PLB and SLN represent a unique set of proteins that bind to and interact with the same SERCA pump but have entirely different functional outcomes.

Mechanism of SLN Uncoupling of SERCA—Although the mechanism of SLN uncoupling remains to be understood, our study represents an important first step toward this goal. During Ca$^{2+}$ transport, the SERCA pump transports two Ca$^{2+}$ ions per molecule of ATP hydrolyzed (1, 55, 56). However, when SLN is bound to SERCA, the energy from ATP hydrolysis does not lead to accumulation of Ca$^{2+}$ in the SR (3, 4), but is released as heat, thus contributing to thermogenesis in muscle (3, 4). Crystallography data of the various SERCA kinetic states show that transferring Ca$^{2+}$ to the SR lumen requires rotation of the A domain of SERCA, which results in the sliding of TM helices that releases Ca$^{2+}$ ions into the SR lumen (1, 57–59). We propose that the interaction of SLN within the SERCA TM groove during Ca$^{2+}$ transport may interfere with TM sliding, resulting in slippage of Ca$^{2+}$ back into the cytosol (60). The MD simulations suggest the possibility that the short and flexible N terminus of SLN is capable of interacting with residues in the SERCA cytosolic domain. Deletion of the SLN N terminus affected spatial orientations of several SERCA residues, which are known to have an influence on SERCA function. Previous studies have
shown that mutations N111A and N114A lead to a reduction in the transport of Ca2+ (61), R324A has the same effect and also inhibits the E1P-E2P transition of SERCA (the step at which Ca2+ is internalized into the SR lumen) (43, 44). Furthermore, SERCA Glu45 lies near the cytosolic portion of the Ca2+ ion binding pathway in SERCA. Although the alanine mutagenesis results for the role of SLN residue Arg3 are inconclusive, its interaction with SERCA Glu45 may play a role in SLN-induced Ca2+ slippage from the pump.

In summary, for the first time we report that the cytosolic N terminus in SLN plays an important role in uncoupling SERCA. The N terminus of SLN may be responsible for ideal positioning of the SLN TM and C terminus in the SERCA groove, to enable uncoupling of the pump. We also show that localization to the SR membrane and interaction in the SERCA groove depends on the SLN TM and C terminus. Our studies highlight the modular nature of the domains in SLN and PLB, which can be interchanged without losing their individual function. Furthermore, we show that when exchanged, the N terminus confers identity to the new protein. We have only begun to understand the role of this region in SLN. Future studies should be aimed at understanding in further detail the interaction of the N-terminal residues of SLN with SERCA.

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The N Terminus of Sarcolipin Plays an Important Role in Uncoupling Sarco-endoplasmic Reticulum Ca\textsuperscript{2+}-ATPase (SERCA) ATP Hydrolysis from Ca\textsuperscript{2+} Transport
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