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Interaction sites among phospholamban, sarcolipin, and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase

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Dedicated to the memory of Professor Setsuro Ebashi.

Abstract

A robust cross-link between Gln^{23} in phospholamban (PLN) and Lys^{328} in the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA1a) is formed in the presence or absence of oxidant and is susceptible to both PLN phosphorylation and SERCA1a Ca^{2+} binding. This cross-link provides precisely the evidence needed to support our earlier proposal that collision of the PLN transmembrane helix at Asn^{27} with the cytosolic extension of M4 at Leu^{321} leads to unwinding of the helix. In a study of site-specific interactions among PLN, sarcolipin (SLN), and SERCA1a, we determined that mutations of some specific amino acids in PLN or SLN diminish either the super-inhibition imposed on SERCA1a function by the PLN–SLN binary complex or the physical interactions between PLN and SLN or both. These results have led to a revision of our earlier model for the PLN–SLN–SERCA1a complex.

Keywords: Ca^{2+}-ATPase; Phospholamban; Sarcolipin; Sarcoplasmic reticulum; Mutagenesis; Molecular structure

Phospholamban (PLN) is a 52-amino acid, integral membrane protein located in the sarcoplasmic reticulum (SR) [1,2]. PLN can interact with and inhibit the activity of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoforms (SERCA1a or SERCA2a), but phosphorylation of PLN at Ser^{16} by cAMP-dependent protein kinase (PKA) and at Thr^{17} by Ca^{2+}/calmodulin-dependent protein kinase (Cam KII) reverses PLN inhibitory function, probably through conformational changes that disrupt protein–protein interactions between PLN and SERCA2a [1,2]. Sarcolipin (SLN) is a 31 amino acid, integral membrane protein [3] that is a functional homologue of PLN [4,5]. NMR structures of both PLN and SLN have been published [6–11].

PLN contains a highly conserved, N-terminal, 30 amino acid, cytosolic sequence, which contains sites for phosphorylation by several kinases [1], whereas SLN has a partly conserved, N-terminal, 7 amino acid cytosolic sequence, which may be phosphorylated at Ser^4 or Thr^5 by kinases such as Serine/Threonine Kinase 16 (STK16) [12]. In the transmembrane sequences of PLN and SLN, 8 amino acids are identical and 8 more are highly conserved as L, I, V or M. Whereas the C-terminus of PLN is hydrophobic, SLN has a highly conserved, hydrophilic, 5 amino acid, C-terminal sequence, Arg-Ser-Tyr-Gln-Tyr^{31}.

SERCAs are 110-kDa transmembrane proteins that transport Ca^{2+} from the cytosol to the SR lumen [13]. High resolution crystal structures for SERCA1a have been determined in five different conformations, accounting for the major steps in the Ca^{2+} transport reaction cycle [14–18]. Of these, only the E2 conformations bind PLN (and, by analogy, SLN) [19,20].

Through mutagenesis and cross-linking, specific amino acids involved in both PLN–SERCA and SLN–SERCA

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interactions were identified throughout the transmembrane sequence and at the cytosolic and luminal boundaries of the membrane [4,5,21–29]. Early studies implicated Lys^3 in PLN [28,30] and amino acids Lys-Asp-Asp-Lys-Pro-Val^{402} [29] in cytosolic interactions, but no cross-links involving Lys^3 were detected in a study using heterobifunctional cross-linking reagents [24]. The Arg-Ser-Tyr-Gln-Tyr^{31} sequence at the C-terminus of SLN was proposed to interact with the luminal surface of SERCA1a [31] and with luminal ER/SR proteins [12]. Hughes et al. [32] used solid-state NMR measurements of local protein dynamics to detect a direct interaction between the Tyr^{29} and Tyr^{31} side groups of SLN and SERCA1a. They also found that addition of the peptide, NAc-Arg-Ser-Tyr-Gln-Tyr, to a functional assay reduced V_{max} for Ca^{2+} transport and inhibited ATP hydrolysis with an IC_{50} of 200 μM.

When PLN and SLN were co-expressed with SERCA, super-inhibition of SERCA activity was observed [4,33]. Super-inhibition was associated with the demonstration that PLN and SLN form a binary complex that is sufficiently stable to result in depolymerization of pentameric PLN [33].

The availability of crystal structures of SERCA1a and the NMR structures of PLN and SLN made it possible for us to model PLN–SERCA1a [20], SLN–SERCA1a [31], and PLN–SLN–SERCA1a [31] interactions within a PLN binding site in SERCA1a, formed dynamically in the E2 conformation as a groove surrounded by transmembrane helices M2, M4, M6, and M9. In these modeling studies, we proposed the most probable interaction face of the binary PLN–SLN complex and deduced that this binary complex interacts with SERCA to create a ternary complex that forms a tighter fit in the regulator-binding groove and has more liganding sites than either a PLN or an SLN monomer alone, thus accounting for its super-inhibitory properties [31]. The groove forming the PLN-binding site was proposed to accommodate PLN, SLN or the predicted PLN–SLN binary complex, but to be too narrow to accommodate two PLN molecules. These modeling studies allowed the prediction of several potential interaction sites among PLN, SLN, and SERCA1a in the transmembrane region.

The first goal of the present study was to test predictions from our modeling by examining whether cytosolic amino acids in PLN and SERCA1a might be located so closely in the PLN–SERCA1a complex that Cys mutant forms would form robust disulfide cross-links. We found a robust cross-link between K328C in SERCA1a and Q23C in PLN. The second goal was to test aspects of our modeling of the interactions among PLN, SLN, and SERCA1a by examining the effects of mutations of specific residues in both PLN and SLN that might be involved in functional or physical interactions among the proteins. Our results confirmed some, but not all, of our predictions from modeling and have led to a revised model.

Materials and methods

Materials. Enzymes for DNA manipulation were obtained from New England Biolabs and Amersham Pharmacia and a chemiluminescence kit for Western blotting was purchased from Pierce. Monoclonal antibody 1D11 against PLN [34] was a gift from Drs. Robert Johnson and Edward McKenna (Merck Research Laboratories). Monoclonal antibody A52 [35] against SERCA1a was made in our laboratory.

Oligoncleotide-directed mutagenesis, expression of mutant cDNA, and preparation of microsomes. The preparation, expression and assay of mutations in PLN, NF-SLN (SLN fused at its N-terminus to the Flag epitope, recognized by the M2 antibody) and SERCA1a in microsome fractions are described in earlier publications [4,21,29].

Site-directed disulfide cross-linking. Disulfide cross-linking was performed as described earlier [20]. Copper phenanthroline was included as an oxidant where indicated. After the cross-linking reaction, the samples were subjected to SDS/PAGE and immunoblotted with antibody 1D11 against PLN. If a 1D11-reactive band was found at a level in the gel slightly higher than the SERCA1a band and if the band was susceptible to reduction by DTT, then cross-linking between PLN and SERCA1a was judged to have taken place. To determine the effect of phosphorylation of PLN by protein kinase A (PKA) on the formation of cross-links, the phosporylation reactions were performed as described in [20] and the samples were then subjected to the cross-linking reaction. To determine the effect of elevated Ca^{2+} on the formation of cross-links, 10 mM $CaCl_2$ replaced 5 mM EGTA and 400 μM Na vanadate in the reaction mixture, thereby inducing the E1Ca2 conformation.

Analsis of Ca^{2+} uptake by the ternary complex. Microsomal fractions from HEK-293 cells expressing SERCA1a alone, or in the presence of PLN or NF-SLN (binary complexes), or in the presence of PLN or PLN mutants plus NF-SLN or NF-SLN mutants (ternary complexes), were assayed for Ca^{2+} transport activity as described previously [36].

Co-immmunoprecipitation of SERCA1a, PLA, and NF-SLN. Microsomes from HEK-293 cells expressing the various ternary complexes described above were solubilized in Tween 20 and co-immunoprecipitation using either antibody 1D11 against PLN or the M2 antibody against the Flag epitope in NF-SLN was performed as described previously [21] and in the legends to Figs. 2 and 3.

Modeling. Modeling was carried out as described previously [20,31].

Results

Analysis of potential interacting sites in cytosolic domains of PLN and SERCA1a

Generation, expression, and detection of Cys mutants of SERCA1a and PLN and measurement of cross-link formation

Cys-substitution mutants of SERCA1a, K328C, T358C, S387C, and N399C and Cys-substitution mutants of PLN, K3C, V4C, L7C, S10C, A11C, I12C, and Q23C were created in order to test their potential involvement in PLN–SERCA1a interaction sites, based on our modeling.

All of the SERCA1a mutants were detected by immunoblotting of microsomal fractions from HEK-293 cells with the A52 antibody against residues 657 and 672 in SERCA1a, and all displayed Ca^{2+} transport activity that was inhibited by PLN. On the other hand, poor 1D11 antibody reactivity with some PLN mutants with a Cys-substitution in the epitope, together with our recognition of the possibility that a cross-link between SERCA1a and N-terminal amino acids in PLN might lie so close to the PLN epitope that interaction with antibody 1D11 would be blocked,

made it difficult to interpret many of our cross-linking results. However, co-expression of SERCA1a mutant K328C with the PLN mutant Q23C showed the formation of a robust, predicted cross-link (Fig. 1A) that was detected by western blotting with antibody 1D11. The PLN Q23C mutant was less inhibitory than wild-type (wt) PLN for both SERCA1a (Table 1A) and for the SERCA1a K328C mutant (Table 1B). In comparison with wt SERCA1a, the SERCA1a K328C mutant manifested both an increase in its apparent Ca^{2+} affinity and in the extent of its inhibition by wt PLN (Table 1B).

The K328C-Q23C cross-link was formed almost as strongly in the absence of the oxidant, copper phenanthroline, as in the presence of the oxidant (Fig. 1A) and it was susceptible to reduction by DTT, applied during the cross-linking reaction (not shown). Both phosphorylation of PLN and the binding of Ca^{2+} to SERCA1a are known to dissociate PLN and SERCA1a, both physically and functionally. The K328C-Q23C cross-link was not formed when the reaction mixture was treated with PKA to phosphorylate PLN at Ser^{16} (Fig. 1B). Similarly, the amount of cross-linking was reduced substantially when the reaction was carried out in 10 mM Ca^{2+} (Fig. 1C). Thus the K328C-Q23C cross-link shares the characteristics of the robust L321C-N27C and V89C-V49C cross-links described earlier [20].

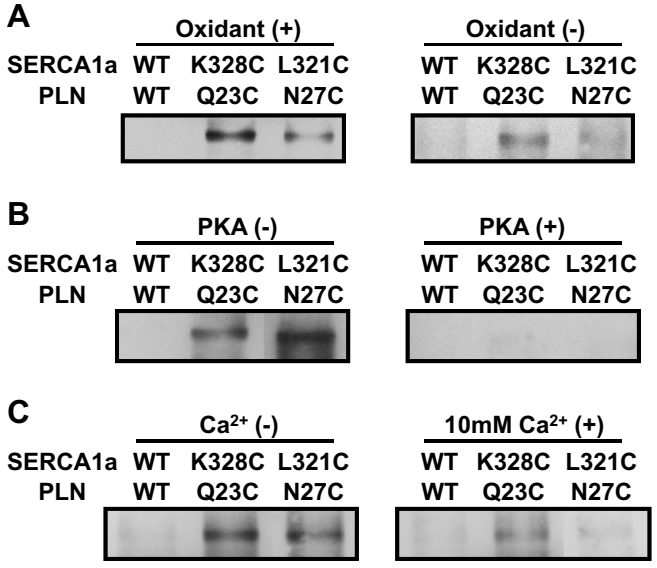

Fig. 1. Characterization of a cross-link between SERCA1a K328C and PLN Q23C. (A) SERCA1a and PLN mutants were co-expressed in HEK-293 cells; microsomal fractions were isolated and subjected to cross-linking conditions, followed by SDS-PAGE and immunoblotting. Staining of a band at about 117 kDa indicates the formation of a strong cross-link between SERCA1a K328C and PLN Q23C. The cross-link was formed in the presence and in the absence of the oxidant, copper phenanthroline. In this experiment and in those presented in B and C, the formation of a cross-link between SERCA1a L321C and PLN N27C mutants was added as a control. (B) The cross-link between SERCA1a K328C and PLN Q23C was abolished by prior phosphorylation of PLN by protein kinase A (PKA). (C) The cross-link between SERCA1a K328C and PLN Q23C was diminished by exposure of SERCA1a to 10 mM Ca^{2+}, which would drive SERCA1a into the E1Ca2 conformation.

Analysis of potential transmembrane interacting sites among PLN, NF-SLN, and SERCA1a

Analysis of functional and physical interactions in the ternary PLN–NF-SLN–SERCA1a complex following mutation of PLN and NF-SLN

To test some of the predictions in our modeling of the ternary PLN–NF-SLN–SERCA1a complex [31], we examined the effects on functional and physical interaction of mutations in several sites in PLN and NF-SLN.

The effects of mutations in PLN on the ability of the PLN–NF-SLN binary complex to inhibit SERCA1a by lowering its apparent affinity for Ca^{2+} are shown in Fig. 2. SERCA1a has high apparent Ca^{2+} affinity, which was lowered by PLN and lowered still further (super-inhibition) by interaction with the binary PLN–NF-SLN complex. When the binary complex included either of PLN mutants I47A or I48A, then the extent of super-inhibition was reduced (Fig. 2A).

The ability of PLN mutants I47A or I48A to disrupt the physical interactions involved in forming the binary PLN–NF-SLN complex was tested by immunoprecipitation of NF-SLN with the M2 antibody, followed by immunoblotting of the complex with antibody 1D11 against PLN. Figs. 2B and C show that mutants I47A, I48A, and I40A all diminished physical interactions between PLN and NF-SLN. In this case, the effect of the PLN I40A mutant did not correlate with the functional data.

The effects of mutations in NF-SLN on the ability of the PLN–NF-SLN binary complex to inhibit SERCA1a by lowering its apparent affinity for Ca^{2+} are shown in Fig. 3. When the binary complex included either of NF-

Table 1
Ca^{2+} affinity of SERCA1a and Cys-substituted SERCA1a mutants in the presence and absence of PLN or Cys-substituted PLN mutants

A:	SERCA1a	SERCA1a + PLN	SERCA1a + PLN Q23C	SERCA1a + PLN K3C
pCa	6.63 ± 0.02	6.27 ± 0.02	6.52 ± 0.01	6.43 ± 0.01
ΔpCa	n.a.	−0.36	−0.11	0.20
B:	SERCA1a K328C	SERCA1a K328C + PLN	SERCA1a K328C + PLN Q23C	
pCa	6.81 ± 0.01	6.35 ± 0.02	6.73 ± 0.05	
ΔpCa	n.a.	−0.46	−0.08	

Ca^{2+} affinity was measured as the EC_{50} (in pCa units) for the Ca^{2+} dependence of Ca^{2+} transport by microsomal fractions from HEK-293 cells expressing SERCA1 ± PLN. ΔpCa is the difference between the pCa value in the presence of PLN compared to its absence. $N = 3$.

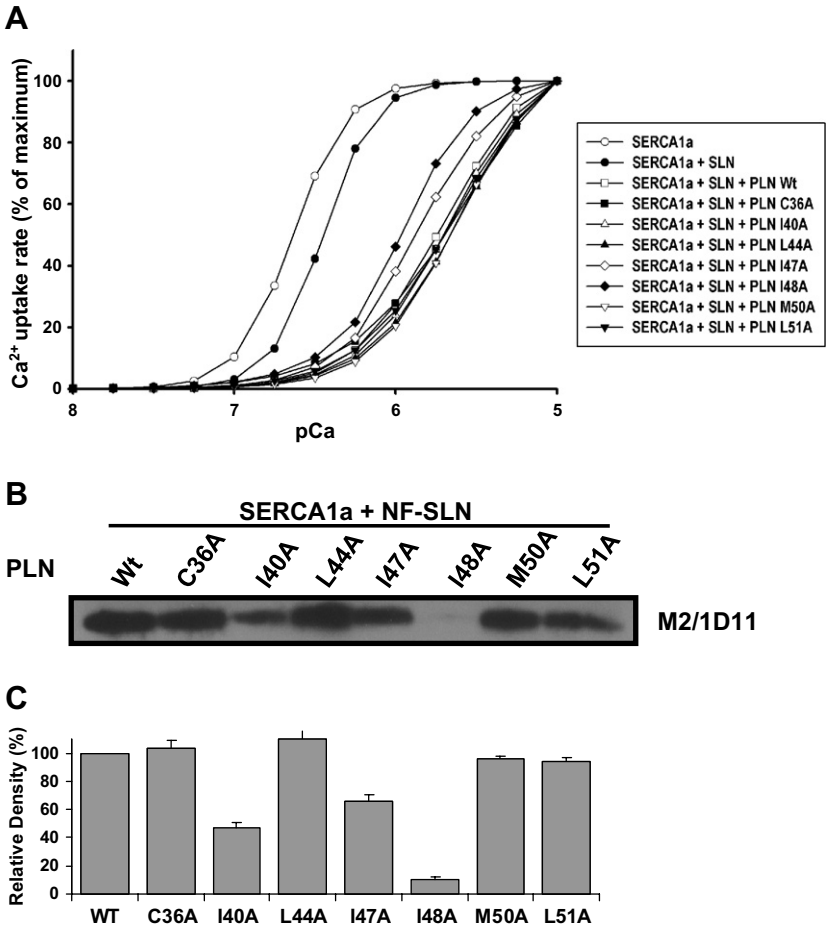

Fig. 2. Effects of mutations in PLN on protein-protein interactions and the level of super-inhibition by binary PLN–NF-SLN complexes. (A) SERCA1a and NF-SLN were co-expressed in HEK-293 cells in the presence of either wt PLN or a series of PLN mutants carrying substitutions in predicted sites of interaction between PLN and SLN. Microsomal fractions were isolated and used for measurement of the Ca^{2+} dependence of Ca^{2+} uptake. PLN mutants I47A and I48A had diminished super-inhibitory capacity. (B) Antibody M2 was used to immunoprecipitate NF-SLN from Tween 20-solubilized extracts of the microsomal fractions, defined in (A). Co-immunoprecipitation of PLN was measured by semiquantitative Western blotting with antibody 1DII against PLN (C). Plots of relative density of bands from (B) show that co-immunoprecipitation was reduced for PLN mutants I40A, I47A, and I48A.

SLN mutants V19A or I22A, then the extent of super-inhibition was reduced (Fig. 3A).

The ability of NF-SLN mutants V19A or I22A to disrupt the physical interactions involved in forming the binary PLN–NF-SLN complex was tested by immunoprecipitation of NF-SLN with the M2 antibody, followed by immunoblotting of the complex with antibody 1D11 against PLN. Figs. 3B and C show that mutants V19A, I22A and W23A all diminished the physical interactions between PLN and NF-SLN. In this case, the effect of the NF-SLN W23A mutant did not correlate with the functional data.

Discussion

Analysis of potential interacting sites in cytosolic domains of PLN and SERCA1a

On the basis of predictions made in our modeling of the binary PLN–SERCA1a complex, it was possible that cross-links might form at a number of sites in the cytosolic domains of PLN and SERCA1a. We showed that a robust cross-link that did not require oxidant and was susceptible to both PLN phosphorylation and SERCA1a Ca^{2+} binding was formed between Gln^{23} and Lys^{328}. This observation is in line with our modeling, which predicts that the distance between the C-γ of Gln^{23} in PLN and that of Lys^{328} in SERCA1a (i.e. the length of a potential S–S bond between Gln^{23}Cys in PLN and Lys^{328}Cys in SERCA1a) could be 2.0 Å, depending on the side chain conformations. It is of particular interest that the K328C–Q23C cross-link provides precisely the evidence needed to support our proposal [20] that the collision of the PLN transmembrane helix at Asn^{27} with the cytosolic extension of M4 at Leu^{321} leads to unwinding of the helix.

Overall, direct cross-linking has provided evidence for three sites of close association between PLN and SERCA1a that include the V89C–V49C cross-link at the luminal-membrane boundary, near the C-terminus of PLN, the L321C–N27C cross-link that lies near the cytosol-membrane boundary, and the cytosolic K328C–Q23C cross-link. The results of extensive studies using a heterobifunctional reagent that cross-links at 10 Å have added consider-

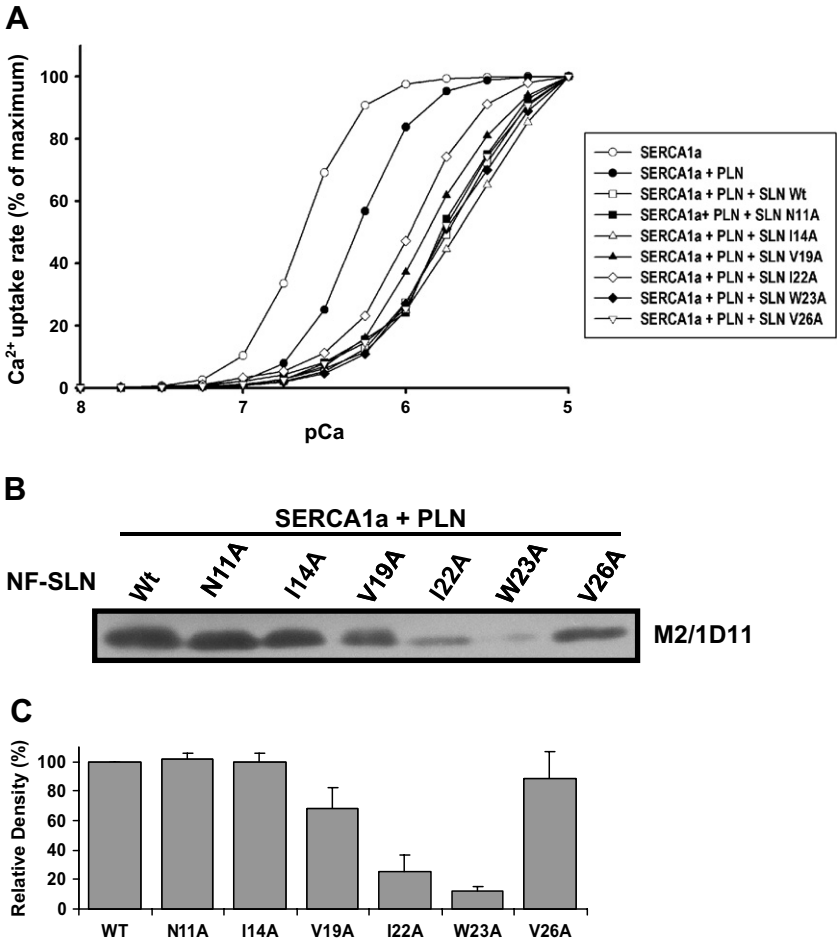

Fig. 3. Effects of mutations in NF-SLN on protein-protein interactions and the level of super-inhibition by binary PLN–NF-SLN complexes. (A) SERCA1a and PLN were co-expressed in HEK-293 cells in the presence of either wt NF-SLN or a series of NF-SLN mutants carrying substitutions in predicted sites of interaction between PLN and SLN. Microsomal fractions were isolated and used for measurement of the Ca^{2+} dependence of Ca^{2+} uptake. NF-SLN mutants V19A and I22A had diminished super-inhibitory capacity. (B) Antibody M2 was used to immunoprecipitate NF-SLN from Tween 20-solubilized extracts of the microsomal fractions, defined in (A). Co-immunoprecipitation of PLN was measured by semiquantitative Western blotting with antibody 1DII against PLN. (C) Plots of relative density of bands from (B) show that co-immunoprecipitation was reduced for NF-SLN mutants V19A, I22A and W23A.

ably to our knowledge of dynamic and spatial aspects of PLN and SERCA2a interactions in the regions lying on either side of the transmembrane bilayer [22–25]. An unresolved question is the site of other cytosolic interactions near the N-terminus of PLN. Although a site involving Lys^3 in PLN (see Table 1A) and Lys-Asp-Asp-Lys-Pro-Val^{402} in SERCA2a has been proposed [28,30,36,37], it has not been confirmed through heterobifunctional cross-linking [24].

Analysis of potential transmembrane interacting sites among PLN, NF-SLN, and SERCA1a

In our analysis of the interactions among PLN, NF-SLN and SERCA1a, we found that mutation of amino acids Ile^{40}, Ile^{47}, and Ile^{48} in PLN and mutation of Val^{19}, Ile^{22} and Trp^{23} in NF-SLN diminished either the super-inhibition imposed on SERCA1a function by the PLN–NF-SLN binary complex or the physical interactions between PLN and NF-SLN or both. On the basis of these results, we propose modifications to our model [31] for the ternary PLN/SLN/SERCA1a complex (Fig. 4). We showed that NF-SLN is able to disrupt PLN pentamers, but does not form homooligomers by itself [33]. These observations mean that the heterodimeric interactions between PLN and SLN are stronger than the homodimeric interactions required for pentamer formation by PLN. It is reasonable to propose that the PLN–SLN complex forms first and then fits into the cavity formed by the M2, M4, M6, and M9 helices of SERCA in the E2 conformation to cause super-inhibition. In our revised model, the hydrophobic side chains of Ile^{40}, Ile^{47}, and Ile^{48} in PLN and Val^{19}, Ile^{22}, and Trp^{23} in SLN lie on the interaction surface between PLN and SLN in the transmembrane region. It is likely that these residues play a key role in forming tight binding between PLN and SLN and can, therefore, influence the formation of the super-inhibitory PLN–SLN–SERCA ternary complex.

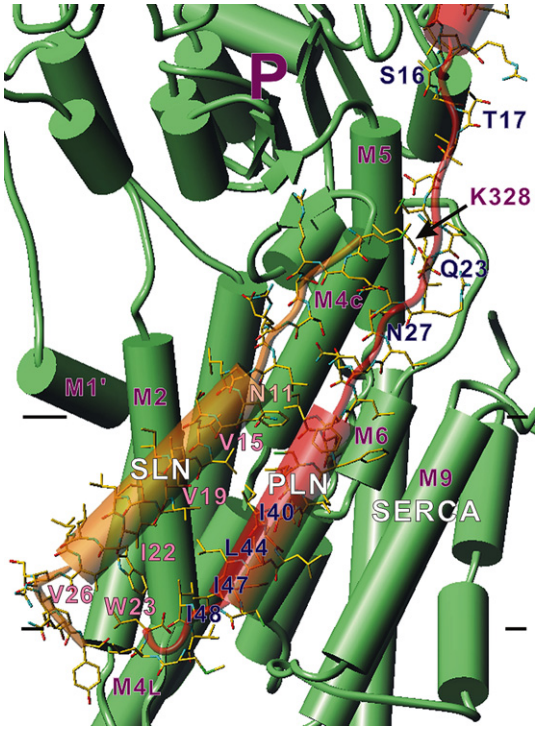

Fig. 4. A model for the ternary PLN–SLN–SERCA1a complex. Details of the interactions between PLN (red) and SLN (orange) are shown. The black arrow indicates the side chain of Lys^{328} in SERCA1a (green). α-helices and β-strands are represented by cylinders and arrows, respectively. Horizontal bars refer to the boundaries of the hydrophobic core of the lipid bilayer.

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