The ability of two loss-of-function mutants, L31A and L31C, of phospholamban (PLB) to bind to and inhibit the Ca\(^{2+}\) pump of cardiac sarcoplasmic reticulum (SERCA2a) was investigated using a molecular cross-linking approach. Leu\(^{31}\) of PLB, located at the cytoplasmic membrane boundary, is a critical amino acid shown previously to be essential for Ca\(^{2+}\)-ATPase inhibition. We observed that L31A or L31C mutations of PLB prevented the inhibition of Ca\(^{2+}\)-ATPase activity and avoided the cross-linking of N27C and N30C of PLB to Ly328 and Cys318 of SERCA2a. Although L31C-PLB failed to cross-link to any Cys or Lys residue of wild-type SERCA2a, L31C did cross-link with high efficiency to T317C of SERCA2a with use of the homobifunctional sulfhydryl cross-linking reagent, 1,6-bismaleimidohexane. This places Leu\(^{31}\) of PLB within 10 Å of Thr317 of SERCA2a, extending into the cytoplasm and highly charged and mostly α-helical. Phosphorylation of either Ser\(^{16}\) or Thr\(^{17}\) within this domain reverses the inhibitory effect of PLB on Ca\(^{2+}\) transport.

The C-terminal domain II (residues 32–52) is a hydrophobic transmembrane helix that contains a Leu/Ile zipper responsible for stabilizing the homopentameric arrangement of PLB in the sarcoplasmic reticulum membrane (9). Although the PLB pentamer comprises a reservoir form of inactive PLB protomers, it is in dynamic equilibrium with a pool of monomeric PLB (10). Monomeric PLB forms a 1:1 complex with SERCA2a (11, 12), and L31I-PLB retained full functional and cross-linking activity, suggesting that a bulky hydrophobic residue at position 31 of PLB is essential for productive interaction with SERCA2a.

Phospholamban (PLB)\(^{1}\) is a 52-amino acid, homopentameric transmembrane protein in the sarcoplasmic reticulum and a key regulator of myocardial contractile kinetics (1–3). Dephosphorylated PLB binds to and inhibits the cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a isoform) by decreasing the apparent affinity of the enzyme for Ca\(^{2+}\) and slowing the E2 to E1 transition of the catalytic cycle of ATP hydrolysis and Ca\(^{2+}\) transport (4). PLB is predicted to have three functional domains (1, 5–8). The N-terminal domain IA (residues 1–20) extends into the cytoplasm and is highly charged and mostly α-helical. Phosphorylation of either Ser\(^{16}\) or Thr\(^{17}\) within this domain reverses the inhibitory effect of PLB on Ca\(^{2+}\) transport. The C-terminal domain II (residues 32–52) is a hydrophobic transmembrane helix that contains a Leu/Ile zipper responsible for stabilizing the homopentameric arrangement of PLB in the sarcoplasmic reticulum membrane (9). Although the PLB pentamer comprises a reservoir form of inactive PLB protomers, it is in dynamic equilibrium with a pool of monomeric PLB (10). Monomeric PLB forms a 1:1 complex with SERCA2a (11, 12), and is thus directly responsible for regulating Ca\(^{2+}\) transport (13, 14).

The intervening domain IB (residues 21–31) of PLB, located at the cytoplasmic border of the sarcoplasmic reticulum membrane, is poorly defined structurally (6) but contains residues with dramatic effects on physical (11, 12) and functional (8) interactions with SERCA2a. As such it appears to be a key region for SERCA2a inhibition. In particular, mutation to alanine of Asn\(^{27}\) or Asn\(^{30}\) of PLB enhanced inhibition of SERCA2a by further decreasing the apparent Ca\(^{2+}\) affinity of the enzyme (8). In contrast, mutation to alanine of Leu\(^{31}\) of PLB totally eliminated inhibitory function (14).

Recently, we developed a chemical cross-linking approach to identify and examine the structural and functional interactions between PLB and SERCA2a, with initial studies suggesting that domain IB of PLB interacts with M4 of SERCA2a (11, 12). In particular, we found that mutation of Asn\(^{27}\) of PLB to cysteine (N27C-PLB) allows strong cross-linking to Lys328 of SERCA2a using the heterobifunctional, 10 Å-long cross-linking agent, EMCS (12). On the other hand, mutation of Asn\(^{30}\) of PLB to cysteine (N30C-PLB) gave strong cross-linking to both Cys318 (11) and Lys328 (12) of SERCA2a with the homobifunctional cross-linking agent, BMH (10 Å cross-linker), and with the heterobifunctional cross-linking agent, KMUS (15 Å cross-linker), respectively. The specificity of PLB to SERCA2a cross-linking was further demonstrated by the absolute requirement for the Ca\(^{2+}\)-free, E2 conformation of SERCA2a, as well as by the consistent effects of the anti-PLB monoclonal antibody, 2D12, and the SERCA2a inhibitor, thapsigargin, in preventing cross-linking (11, 12).

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Leu31 is another critically important residue in domain IB of PLB that is required for PLB function. Changing Leu31 of PLB to Ala (L31A-PLB) abolishes its inhibitory capacity (14). Kimura et al. (14) and Asahi et al. (15) postulate that the loss-of-function exhibited by a class of mutants including L31A-PLB is due to their inability to bind to SERCA1a in the membrane-spanning region. In the case of Leu31, however, other evidence is consistent with localizing this residue at the cytoplasmic membrane interface within domain IB (6, 16, 17). A subsequent structural model by Toyoshima et al. (18) suggests that Leu31 of PLB interacts directly with Thr805 and Phe809 in previously (11–13, 20). Microsomes (48,000 g canine isoforms) was conducted in Sf21 insect cells as described previously (11, 12). All mutated cDNAs were confirmed by DNA sequencing. Baculovirus infection of pVL1393 containing mutated cDNAs into Sf21 insect cells with materials reported are Ca2+ pump, consistent with our previous results on cross-linking of N27C-PLB and N30C-PLB to WT-SERCA2a (11, 12). Like the other two domain IB residues, Asn27 and Asn30, Leu31 of PLB appears to interact with M4 on SERCA2a, not M6 as recently proposed by Toyoshima et al. (18). Possible mechanisms for the important role of Leu31 in domain IB of PLB are discussed.

EXPERIMENTAL PROCEDURES

Materials—The homobifunctional sulfhydryl cross-linking agent, BMH, and the heterobifunctional amine-sulfhydryl cross-linking agents, EMCS and KMUS, were obtained from Pierce. Thapsigargin was purchased from Sigma. Iodogen was from Pierce.

Mutagenesis and Baculovirus Production—Mutagenesis of canine SERCA2a and PLB cDNAs was conducted as recently described (11, 12). PLB mutagenesis was conducted on the Cys-less PLB background, which is canine PLB with Cys residues 36, 41, and 46 replaced by Ala (11, 12). All mutated cDNAs were confirmed by DNA sequencing. Baculoviruses encoding mutated proteins were generated by co-transfection of pVL1393 containing mutated cDNAs into SF21 insect cells with BaculoGoldTM (Pharmlingen) linearized baculovirus DNA (11–13, 20).

Protein Co-expression in Insect Cells and Isolation of Microsomes—Co-expression mutants with WT-SERCA2a or T317C-SERCA2a (canine isoforms) was conducted in SF21 insect cells as described previously (11–13, 20). Microsomes (48,000 × g) were isolated 60 h after initiating baculovirus infections and stored frozen in small aliquots at −40 °C. Protein concentration was determined by the Lowry method (13).

Ca2+-ATPase Assay—Ca2+-ATPase activities of insect cell microsomes co-expressing PLB and SERCA2a were measured at 37 °C in the presence and absence of the anti-PLB antibody, 2D12, in buffer containing 50 mM MOPS (pH 7.0), 3 mM MgCl2, 100 mM KCl, 5 mM NaN3, 3 μg/ml A23187, 3 mM ATP, and 1 mM EGTA (13, 20). Ionized Ca2+ concentration was varied by adding CaCl2 (13, 20). All ATPase activities reported are Ca2+-dependent.

Cross-linking—Cross-linking between Cys-substituted residues of PLB and residues of SERCA2a in insect cell microsomes was conducted at room temperature as described recently, using a final concentration of cross-linking reagent of 0.1 mM (11, 12). Reactions were started by adding 0.75 μl of concentrated cross-linking reagent in dimethyl sulfoxide to 11 μg of micromolar protein in 12 μl of buffer A, which consisted of 40 mM MOPS (pH 7.0), 3.2 mM MgCl2, 75 mM KCl, 3 mM ATP, and 1 mM EGTA. Reactions were terminated by adding 7.5 μl of SDS-PAGE sample loading buffer containing 15% SDS and 100 mM dithiothreitol. The samples were then subjected to SDS-PAGE and immunoblotting. When the heterobifunctional (Cys to Lys) cross-linking reagents, EMCS and KMUS, were used, reactions were generally conducted for 10 min at room temperature. With use of the homobifunctional (Cys to Cys) cross-linker, BMH, reactions were conducted for 30–60 min at room temperature (except in Fig. 6).

RESULTS

L31A Mutation Prevents PLB Cross-linking to WT-SERCA2a—Using thiol- and amine-selective cross-linking agents, we previously demonstrated that residues 27 and 30 in domain IB of PLB cross-link with high specificity to Lys325 and Cys318 of SERCA2a at the cytoplasmic extension of M4 (11, 12). N27C-PLB cross-linked to Lys325 of WT-SERCA2a most efficiently with the heterobifunctional cross-linking agent, EMCS, which has a length of 10 Å (Fig. 1A, left panel) (12), whereas N30C-PLB cross-linked most efficiently to Lys318 of WT-SERCA2a with the heterobifunctional cross-linking agent, KMUS, with a length of 15 Å (Fig. 1C, left panel) (12). In addition, N30C-PLB cross-linked to Cys316 of WT-SERCA2a with the homobifunctional cross-linking agent, BMH, with a length of 10 Å (Fig. 1C, left panel) (11). Like WT-PLB (13), N27C-PLB and N30C-PLB both inhibit the Ca2+-ATPase activity of WT-SERCA2a by shifting the Ca2+ activation curve to the right, giving KCa values of 0.22 and 0.27 μM, respectively (Fig. 1, B and D, open squares). Addition of the anti-PLB monoclonal antibody, 2D12, which mimics the effect of PLB phosphorylation (4), reverses this inhibition, giving KCa values of 0.12 and 0.15 μM, respectively (Fig. 1, B and D, closed squares).

Directly adjacent to Asn30 of PLB, Leu31 is at the cytoplasmic boundary of the membrane (6) and appears to be essential for PLB function (14, 15). To clarify the role of Leu31 of PLB, we first confirmed the original observation of Kimura et al. (14) that replacement with the smaller residue alanine (i.e. L31A) abolishes PLB inhibition of Ca2+-ATPase activity and Ca2+ transport (data not shown). To explain this result, Kimura et al. hypothesized that L31A-PLB exhibits a decreased binding affinity for SERCA2a (14), an idea that was supported in a subsequent co-immunoprecipitation study (15). To further test this hypothesis, we co-expressed two double mutants of PLB, namely, N27C,L31A-PLB and N30C,L31A-PLB, with WT-SERCA2a and checked for loss of cross-linking function. As predicted, when the L91A mutation was introduced into PLB, neither N27C nor N30C of PLB retained the ability to cross-link to Lys325 of WT-SERCA2a, using EMCS and KMUS, respectively (Fig. 1, A and C). Likewise, the L31A mutation prevented the cross-linking of N30C of PLB to Cys316 of WT-SERCA2a by BMH (Fig. 1C). Furthermore, neither of the two PLB double mutants, N27C,L31A-PLB nor N30C,L31A-PLB, was able to inhibit the Ca2+-ATPase activity of WT-SERCA2a (Fig. 1, B and D, open and closed circles). These results appear to confirm the idea that the L31A mutation impairs the binding of PLB to WT-SERCA2a and thus the ability of PLB to inhibit Ca2+ pump activity.

Failure of L31C-PLB to Cross-link to WT-SERCA2a—To investigate further whether Leu31 of PLB is essential for physical association with WT-SERCA2a and thereby inhibition of its ATPase activity, we made the L31C-PLB mutant and tested for
FIG. 1. **L31A effect on cross-linking and Ca\(^{2+}\)-ATPase activity.** A, 11-μg aliquots of insect cell microsomes co-expressing N27C-PLB and WT-SERCA2a (left) or N27C,L31A-PLB and WT-SERCA2a (right) were incubated with 0.1 mM cross-linking agents in buffer A for 10 min (EMCS or KMUS) or 1 h (BMH) at room temperature (top). Reactions were terminated with sample loading buffer, samples subjected to SDS-PAGE and
Efficient Cross-linking of L31C-PLB to T317C-SERCA2a—The failure to obtain cross-linking between Leu31 mutants of PLB and WT-SERCA2a appears to support the hypothesis that L31C (or A)-PLB is incapable of binding to SERCA2a. However, the possibility remained that these PLB mutants could still bind to the Ca\(^{2+}\) pump but that residue 31 is sterically hindered from efficiently cross-linking to endogenous Cys or Lys residues of WT-SERCA2a. Indeed, the cross-linked residues of SERCA2a identified in this region (11, 12), Cys\(^{318}\) appears to be buried on the wrong side of the M4 helix in x-ray crystallographic structures of SERCA (21, 22), and Lys\(^{328}\) is over 15 Å away from PLB residue 31 at the upper end of M4 (see Fig. 8). We reasoned that Leu\(^{31}\) of PLB might be considerably closer to Thr\(^{317}\) of SERCA2a, which is located one residue below Cys\(^{318}\) and on the outside face of the M4 helix. To further test for a potential interaction between Leu\(^{31}\) on PLB and M4 on SERCA2a, we made the T317C mutant and checked for cross-linking of T317C-SERCA2a to L31C-PLB. Indeed, strong cross-linking of L31C-PLB to T317C-SERCA2a was observed using the 10 Å-long, homobifunctional thiol-reactive cross-linking agent, BMH (Fig. 3A). At the same time, L31C-PLB failed to cross-link to Lys\(^{328}\) of T317C-SERCA2a using the heterobifunctional cross-linking agents, EMCS and KMUS (Fig. 3A), confirming results obtained with WT-SERCA2a (Fig. 2) and supporting the notion of spatial constraints as an explanation for the lack of cross-linking between Leu\(^{31}\) of PLB and Lys\(^{328}\) (or Cys\(^{318}\)) of SERCA2a. Despite the implication of strong binding between L31C-PLB and T317C-SERCA2a as indicated by the

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**Fig. 2. L31C effect on cross-linking and Ca\(^{2+}\) -ATPase activity.** A, L31C-PLB (L31C) was co-expressed with WT-SERCA2a, and membranes were checked for cross-linking between the two proteins as described in the Fig. 1 legend. B, cross-linking between N27C-L31C-PLB and N30C,L31C-PLB co-expressed with WT-SERCA2a. C, cross-linking between N30C-L31C-PLB and N30C,L31C-PLB co-expressed with WT-SERCA2a. D, Ca\(^{2+}\) -ATPase activities of corresponding microsomes measured in the absence (−) or presence (+) of the anti-PLB antibody. K\(_{Ca}\) values (−) antibody were: L31C-PLB, 0.14 (−) and 0.13 μM (+); N27C,L31C-PLB, 0.14 (−) and 0.13 μM (+); N30C,L31C-PLB, 0.15 (−) and 0.13 μM (+). Similar results were obtained in three separate assays.

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immunoblotting, and blots probed with anti-PLB monoclonal antibody, 2D12. Control samples (CON) had no cross-linker added. PLB/SER, PLB cross-linked to WT-SERCA2a; PLB\(_1\), and PLB\(_2\), PLB monomer and dimer. All PLB mutants were expressed on the Cys-less PLB background. B, Ca\(^{2+}\) -ATPase activities of insect cell microsomes co-expressing WT-SERCA2a and corresponding PLB mutants in panel A. ATPase activities were measured in the absence (−) or presence (+) of the anti-PLB antibody, 2D12. C, cross-linking of N30C-PLB (left) and N30C,L31A-PLB (right) to WT-SERCA2a, conducted as described in panel A. D, Ca\(^{2+}\) -ATPase activities of insect cell microsomes co-expressing WT-SERCA2a and corresponding PLB mutants in panel C. See “Results” for K\(_{Ca}\) values obtained from panels B and D. The K\(_{Ca}\) value for SERCA2a expressed by itself in microsomes was 0.15 ± 0.03 μM (n = 4) when Ca\(^{2+}\) -ATPase activities were determined in the presence or absence of the anti-PLB antibody in the same series of experiments.
strong PLB cross-linking signal at 110 kDa obtained with BMH (Fig. 3A), L31C-PLB was incapable of inhibiting ATP hydrolysis by T317C-SERCA2a over the entire range of ionized Ca\(^{2+}\) concentrations tested (Fig. 3B, open and closed squares), consistent with results using WT-SERCA2a (Fig. 2D). All of these results suggest that L31C (or A)-PLB binds strongly to SERCA2a near residue 317 but is nevertheless devoid of inhibitory function.

A variety of tests were made to verify that the Ca\(^{2+}\) pump was not adversely affected by the T317C point mutation. First, T317C-SERCA2a and WT-SERCA2a exhibited similar Ca\(^{2+}\)-ATPase activities and identical \(K_{Ca}\) values for ATPase activation when expressed alone (data not shown). Second, T317C-SERCA2a was inhibited normally by PLB lacking the L31C mutation, and this inhibition was completely reversed by the anti-PLB antibody (Fig. 3B, open and closed circles). Third, we checked for cross-linking of T317C-SERCA2a to N27C-PLB and N30C-PLB, the two gain-of-function PLB mutants (Fig. 4). As expected, the heterobifunctional cross-linking agents, EMCS and KMUS, efficiently cross-linked N27C-PLB and N30C-PLB, respectively, to T317C-SERCA2a (Fig. 4B). Likewise, BMH efficiently cross-linked N30C-PLB (as well as N27C-PLB) to T317C-SERCA2a (Fig. 4B). In contrast, L31C-PLB was only capable of cross-linking to T317C-SERCA2a (not WT-SERCA2a) and only with BMH (Fig. 4, middle panels). Fourth, we also observed that N27C-PLB and N30C-PLB inhibited the Ca\(^{2+}\)-ATPase activity of T317C-SERCA2a like WT-SERCA2a (data not shown). Therefore, the T317C-SERCA2a mutant appears to be a useful surrogate of WT-SERCA2a for assessing PLB and SERCA2a physical and functional interactions.

We also noted that like cross-linking to WT-SERCA2a (Fig.

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**Fig. 3.** L31C-PLB interactions with T317C-SERCA2a. A, cross-linking of L31C-PLB to T317C-SERCA2a \((L31C/T317C)\) conducted as described in Fig. 1 legend. B, Ca\(^{2+}\)-ATPase activities of membranes used in panel A, as well as of insect cell microsomes co-expressing Cys-less PLB and T317C-SERCA2a. \(K_{Ca}\) values were 0.35 and 0.18 \(\mu M\) for Cys-less PLB co-expressed with T317C-SERCA2a (open and closed circles, respectively, representing activities measured in the absence or presence of the anti-PLB antibody, respectively) and 0.20 and 0.18 \(\mu M\) for L31C-PLB co-expressed with T317C-SERCA2a (open and closed squares, in the absence or presence of antibody, respectively). Similar results were obtained in four separate assays. In other experiments, the \(K_{Ca}\) values for T317C-SERCA2a co-expressed with WT-PLB were 0.31 ± 0.04 and 0.20 ± 0.02 \(\mu M\) when Ca\(^{2+}\)-ATPase activities were measured in the absence or presence of anti-PLB antibody, respectively \((n = 3)\).

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**Fig. 4.** Comparison of cross-linking of different PLB mutants to WT-SERCA2a (A) and to T317C-SERCA2a (B). Cross-linking was conducted as described in the Fig. 1 legend. Cross-linkers utilized are indicated at top. PLB mutants cross-linked to SERCA2a are indicated on the left. For economy of space, only PLB/SERCA2a cross-linked bands on autoradiographs are shown in this and remaining figures.
inhibition of cross-linking at other sites reported previously (11, 12) and suggests that PLB dissociates from SERCA2a at this site once the enzyme has bound Ca

Thapsigargin, the irreversible inhibitor of SERCA, abolished the cross-linking of L31C-PLB to T317C-SERCA2a with $K_{i}$ of $0.13 \pm 0.02 \mu M$ (Fig. 5B), supporting our conclusion that PLB is incapable of binding to E2-thapsigargin (11, 12). In the same experiments, the concentration of SERCA2a in the cross-linking assay was estimated to be $0.2-0.3 \mu M$ by quantitative immunoblotting (11), confirming the earlier observation that thapsigargin titrates the Ca

Allosteric Regulation of Cross-linking of L31C-PLB to T317C-SERCA2a—Allosteric Regulation of cross-linking of L31C-PLB to T317C-SERCA2a was virtually eliminated by the L31C mutation (Fig. 4B, two bottom panels). However, in this instance, the N27C or N30C PLB mutations reciprocated by also preventing the cross-linking of L31C of PLB to T317C of SERCA2a.

**Allosteric Regulation of Cross-linking of L31C-PLB to T317C-SERCA2a—** Allosteric agents markedly affect the cross-linking of PLB to SERCA2a. We previously demonstrated that N27C-PLB and N30C-PLB cross-link most efficiently to WT-SERCA2a when the enzyme is in the E2, nucleotide-bound state. Moreover, specific cross-linking was prevented by the irreversible SERCA inhibitor, thapsigargin, as well as by the anti-PLB monoclonal antibody, 2D12, which reverses SERCA2a inhibition like PLB phosphorylation (11–13, 20). To confirm the conformational requirement for covalent coupling of L31C-PLB to T317C-SERCA2a, effects of allosteric agents on cross-linking at this site were tested.

Micromolar Ca

which drives SERCA2a to the E1 conformation, totally inhibited cross-linking of L31C-PLB to T317C-SERCA2a by BMH (Fig. 5A). A $K_{i}$ value of $0.38 \pm 0.03 \mu M$ was obtained, which is consistent with $K_{i}$ values for Ca

FIG. 5. Allosteric effectors on cross-linking of L31C-PLB to T317C-SERCA2a with BMH. Cross-linking was conducted for 10 min at room temperature in buffer A with 0.1 mM BMH. A, L31C-PLB/T317C-SERCA2a cross-linking signals (inset, for example) obtained at different ionized Ca

concentrations were quantified and plotted (graph) as percentages of the cross-linking signal obtained at zero added Ca

$K_{i}$ concentration of SERCA2a in the cross-linking assay was estimated to be $0.2-0.3 \mu M$ by quantitative immunoblotting (11), confirming the earlier observation that thapsigargin titrates the Ca

Allosteric effectors on cross-linking of L31C-PLB to T317C-SERCA2a (Fig. 5C), again consistent with our previous finding that domain IB residues of PLB interact most productively with SERCA2a when it is in the nucleotide-bound, E2 conformation (11, 12). Half-maximal stimulation of cross-linking occurred at 29.0 ± 3.0 $\mu M$ for ATP and at 50.3 ± 8.7 $\mu M$ for ADP (mean ± S.D. from three determinations), confirming the nucleotide affinities of E2 previously estimated from cross-linking N30C of PLB to Cys

of SERCA2a with BMH (11). It should be noted that the affinity for ATP for the E2 state of SERCA is reduced by two orders of magnitude by thapsigargin, as reported by DeJesus et al. (24). Finally, the anti-PLB monoclonal antibody, 2D12, completely eliminated cross-linking of L31C-PLB to T317C-SERCA2a (Fig. 5D), whereas the anti-SERCA2a monoclonal antibody, 2A7-A1, which has no effect on PLB function or SERCA2a activity (12), also had no effect on cross-linking of L31C-PLB to T317C-SERCA2a (Fig. 5D). All of these results demonstrate that defined conformations of SERCA2a and PLB are essential for cross-linking at multiple sites throughout both molecules (11, 12).

Time Course of Cross-linking of L31C-PLB to T317C-SERCA2a—Cross-linking of N30C-PLB to Cys

of WT-SERCA2a in the presence of BMH is a slow process occurring with a $t_{1/2}$ of ~15 min at room temperature (11) (Fig. 6). The slowness of the reaction was attributed to poor accessibility of Cys

of SERCA2a, which appears buried in the interface between M4, M5, and M6, when SERCA2a is in the E2 conformation (see Fig. 8). In contrast, cross-linking of the adjacent residue, L31C of PLB, to the adjacent residue, T317C of SERCA2a, by BMH is a much faster process, occurring with a $t_{1/2}$ of 110 s at room temperature (Fig. 6) and with a $t_{1/2}$ of 55 s at 37°C (data not shown). Thus, in comparison to residue 318 of SERCA2a, residue 317 appears more spatially accessible and better oriented to allow rapid cross-linking to PLB.

L31I-PLB Retains Cross-linking Function and Ability to Inhibit Ca

-ATPase—Replacement of Leu

of PLB with either of the 2 amino acids containing short hydrocarbon side chains (L31A or L31C) completely abrogated the ability of PLB to cross-link to WT-SERCA2a and to inhibit Ca

-ATPase activity (Figs. 1 and 2). We wondered whether substitution of the comparably hydrophobic isomer, Ile, for Leu

would preserve PLB cross-linking function and inhibition of ATPase activity. Indeed, N27C,L31I-PLB cross-linked to WT-SERCA2a as strongly as the single point mutant, N27C-PLB, with EMCS being the most efficient cross-linking reagent for both PLB mutants (Fig. 7A). Likewise, N30C,L31I-PLB cross-linked to WT-SERCA2a as strongly as the single point mutant, N30C-PLB; in this case, BMH and KMUS were the most efficient cross-linkers for both PLB mutants (Fig. 7B). Both double mutants also inhibited Ca

-ATPase activity by decreasing the apparent affinity of the enzyme for Ca

, and inhibition was reversed by the anti-PLB antibody (Fig. 7, C and D). In other experiments, we observed that the single point mutant, L31I-PLB, was equally effective as WT-PLB in inhibiting Ca

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Close Interaction between L31C of PLB and T317C of SERCA2a

ATPase activity (data not shown). Thus, a long hydrocarbon side chain at residue 31 of PLB appears to be sufficient for retention of PLB structural integrity and inhibition of SERCA2a enzymatic activity.

**DISCUSSION**

To gain a better understanding of the structural and functional interactions that govern inhibition of SERCA2a by PLB, we initiated a series of cross-linking studies to localize functionally important amino acid residues that participate in SERCA2a regulation (11, 12). Using homobifunctional and heterobifunctional cross-linking agents as molecular rulers, we previously mapped the distances between N27C and N30C in domain IB of PLB and Cys318 and Lys328 along M4 of SERCA2a and determined the key allosteric factors that affect the interaction of these residues during the catalytic cycle (11, 12). Asn27 and Asn30 of PLB, when changed to Ala, produce gain-of-function effects characterized by enhanced inhibition of SERCA2a activity (8). Here we have shown that Leu31, which produces loss-of-function when mutated to Cys or Ala (14), can also be mapped relative to M4 on SERCA2a. In particular, we were successful in cross-linking L31C-PLB to T317C-SERCA2a using the homobifunctional thiol cross-linking agent, BMH, but not to WT-SERCA2a, using a variety of homobifunctional and heterobifunctional reagents. Furthermore, like previous results observed at Asn27 and Asn30 of PLB, the cross-linking at L31C of PLB occurred only in the absence of Ca2+ and in the presence of nucleotide and was completely inhibited by thapsigargin or by the anti-PLB monoclonal antibody that mimics the physiological effects of phosphorylation (25). Taken together, these data encompassing two gain-of-function PLB mutants and one loss-of-function PLB mutant strongly support our hypothesis that domain IB of PLB interacts with SERCA2a exclusively in the Ca2+-free, E2 conformation, preferentially with bound nucleotide, and that PLB does not interact with the thapsigargin-inhibited, E2 state (11, 12).

The highly efficient cross-linking of L31C-PLB to T317C-SERCA2a demonstrated for the first time that L31C-PLB, though devoid of inhibitory function, still actively binds to SERCA2a. Using alanine scanning mutagenesis, Kimura et al. first proposed that Leu31 of PLB interacts directly with SERCA2a and that substitution with alanine at this position disrupts PLB binding to the Ca2+ pump (8, 14). This conclusion was supported by results from a subsequent co-immunoprecipitation study by Asahi et al. (15). However, the results presented here directly contradict the idea that Leu31 mutants of PLB dissociate from SERCA2a and that inhibitory activity necessarily correlates with binding affinity. In particular, BMH cross-linked L31C-PLB to T317C-SERCA2a eight times more rapidly and with similar yield compared with its cross-linking of N30C-PLB to WT-SERCA2a, where up to 40% of the SERCA2a molecules were coupled (11). Thus, if inhibitory capacity of PLB could be explained simply by its ability to bind to the Ca2+ pump, one would expect L31C, L31A, N30C, and N30A mutations all to be functional or to cause gain-of-function. Based on the loss-of-function actually observed for the Leu31 mutations, we instead suggest that although the L31C (and presumably L31A) PLB mutants are able to bind to SERCA2a, the resulting complexes cannot inhibit Ca2+-ATPase activity.

Our results with the PLB double mutants, N27C,L31A (or C) and N30C,L31A (or C), suggest that Leu31 plays a determinisitic role in productive associations between PLB and SERCA2a that overrides the interactions at Asn27 and Asn30. We note that L31C-PLB, the single point mutant, cross-linked strongly to T317C-SERCA2a even though all inhibitory function was lost. When coupled with mutations at Asn27 and Asn30, L31C or L31A disrupted the ability of PLB residues 27 or 30 to cross-link to either Cys318 or Lys328 of WT-SERCA2a and also abolished all inhibition of the Ca2+ pump normally associated with these gain-of-function Asn mutations (Figs. 1, 2, and 4). Thus, Leu31 appears to provide the more dominant interaction with SERCA2a, although Asn27 and Asn30 also contribute, because both of the double mutants, N27C,L31C-PLB and N30C,L31C-PLB, also failed to cross-link to T317C-SERCA. A productive inhibitory interaction was shown to require a bulky hydrophobic residue at position 31, because either Leu or Ile are tolerated but cannot be substituted by either Ala or Cys.

Modeling of the structural interactions between PLB and SERCA2a at this region also supports the involvement of Leu31 of PLB in a critical interaction with SERCA2a that is responsible for both inhibitory effect and for facilitating cross-linking to nearby residues. We have built a model using an α-helical PLB molecule derived from an NMR structure (26) juxtaposed with the x-ray structure of SERCA1a in the presence of thapsigargin and EGTA (22) to try to account for the cross-linking results obtained to date (Fig. 8). Although there is reason to believe that neither of these structures corresponds strictly to the physiological conformation of the inhibitory complex (see below), it is nevertheless instructive for potential interactions. Inspection of the SERCA1a structure shows a hydrophobic patch at the cytoplasmic membrane surface formed by Gly808 and Phe809 at the top of M6 and Leu821, Cys818, and the methyl group from Thr817 on M4. This patch would be suitable for interaction with Leu31 and Leu28 (8) of PLB. To maximize these associations, we rotated the PLB helix by ~60° relative to our earlier model (12). In addition to explaining the mutational sensitivity of Leu31 and Leu28 (8, 14), this orientation is con-

**FIG. 6. Time course of cross-linking.** Insect cell microsomes co-expressing N30C-PLB and WT-SERCA2a (open squares) (N30C/WT) or L31C-PLB and T317C-SERCA2a (closed squares) (L31C/T317C) were incubated with 0.1 mM BMH in buffer A at room temperature for the times indicated, and cross-linking of PLB to SERCA2a was then determined. The insets show the anti-PLB immunoblots obtained after cross-linking, and the graph shows the results plotted.
consistent with cross-linking both of L31C-PLB with T317C of SERCA2a and of V49C-PLB with V89C-SERCA2a (18). However, this orientation is inconsistent with recent three-dimensional models of Toyoshima et al. (18) and Hutter et al. (27) in which residue 31 of PLB points in the opposite direction, toward M9. Asn27 and Asn30 of PLB are also quite close to Leu321 of SERCA, as suggested by disulfide formation between N27C-PLB and N30C-PLB and L321C of SERCA2a (18). However, Asn27 and Asn30 have moved farther away from Cys318 of SERCA2a, which is consistent with the slower rate of cross-linking of N30C-PLB to Cys318 of SERCA2a (11), compared with cross-linking these Asn mutants to Lys328 (12) and L321C (18). This lower rate of reactivity at Cys318 also reflects steric constraints due to the location of Cys318 on the buried face of M4.

It appears likely that the native structures of both PLB and SERCA vary to some degree from those depicted (Fig. 8). In the case of SERCA, crystallization required the presence of thapsigargin and occurred in the absence of ATP, which makes the structure incompatible with two counts with the cross-links to T317C (this study), Cys318 (11), and Lys326 (12). Thapsigargin is well known to trap SERCA in a rigid, dead-end complex that is derived from the E2 conformation (28), which may involve locking Cys318 in the buried location seen in this structure. Thus, the particular configuration of residues forming the hydrophobic patch between M4 and M6 may be different in the E2 state not inhibited by thapsigargin or may benefit from flexibility not allowed by thapsigargin. Indeed, comparison of this structure with that formed in the presence of Ca^{2+}/H11001 reveals major changes of both M4 and M6, thereby illustrating the dynamic nature of this region of the pump and suggesting that the accessibility of Cys318 to cross-linking may reflect either differences in structure or dynamics of M4 in the absence of thapsigargin. Furthermore, we can conclude that Ca^{2+} binding to SERCA will replace the hydrophobic patch at the cytoplasmic membrane interface of M4 and M6 with a surface of different character, very likely contributing to the displacement of PLB from its binding site and explaining the cross-linking requirement for the Ca^{2+/-}free state of SERCA2a.

In the case of PLB, the model does not explain why L31C cannot cross-link to Cys318 and, given the unphysiological con-
ditions used for the NMR measurements (nonpolar solvent and lack of SERCA2a interaction) (26), changes to the cytoplasmic domain of PLB in this model can also be contemplated. One possibility is that Leu31 is constrained by its participation in the transmembrane helix but that this helix becomes unwound beyond Leu31, which indeed was suggested in earlier models (6, 16, 18). The model of Tatulian et al. (16) includes a short anti-parallel, two-strand β-sheet that runs parallel to the membrane surface and that includes both Asn 27 and Asn30. Unlike the highly extended chain in the model of Toyoshima et al. (18), this short sheet would keep Asn 30 rather near Cys 318 and potentially maintain the 5 Å differential observed in cross-linking these Asn residues to Lys 328 of SERCA. This β-sheet would likely exclude interaction between Lys 3 of PLB and the KDDK-400 loop of SERCA2a, but the early cross-linking suggested at these sites (29) was not reproducible in later studies (12).

The results of cross-linking and mutagenesis make clear that the region of PLB at the membrane surface is crucial in defining the functional interaction with SERCA2a. This is likely to reflect a particular surface that is presented by SERCA2a in the E2, Ca\(^{2+}\)-free conformation and that is recognized by the portion of PLB that includes residues 27–31. The idea of a hydrophobic surface on SERCA2a binding PLB is consistent with mutagenesis results showing that mutation of the Asn residues of PLB to either Ala or Cys at the interaction site enhances the inhibition. The mechanism of PLB inhibition has been attributed to a slowing of the conformational change during binding of Ca\(^{2+}\) to SERCA2a (4). From a structural point of view, this could be explained by PLB binding to the E2 conformation of the pump and physically resisting the movements of M4 and M6 that are required to bind Ca\(^{2+}\), as well as the dramatic tilting of M2 that accompanies the transition to the E1 conformation (21, 22).

In summary, our data on cross-linking the loss-of-function PLB mutant, L31C, to T317C-SERCA2a further demonstrate the power of cross-linking agents as molecular rulers to reveal important information on structural interactions between integral membrane proteins at a high level of resolution. Cross-linking agents also allow functional issues to be addressed, in this case by providing an opportunity to assess effects of allosteric regulators required for SERCA2a activity on protein-protein interactions occurring in the membrane.
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REFERENCES