Phospholamban Domain Ib Mutations Influence Functional Interactions with the Ca^{2+}-ATPase Isoform of Cardiac Sarcoplasmic Reticulum*

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Alanine-scanning mutagenesis of amino acids 21–30, forming cytoplasmic domain Ib in phospholamban (PLN), revealed that mutation to Ala of Asn^{27}, Gln^{29}, and Asn^{30} results in gain of inhibitory function. In an earlier study (Kimura, Y., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1997) J. Biol. Chem. 272, 15061–15064), gain of function in PLN transmembrane domain II mutants was correlated with pentamer destabilization, leading to proposals that the PLN monomer is the active inhibitory species, that dissociation of the PLN pentamer is one determinant of PLN inhibitory function and that dissociation of the PLN-cardiac sarco(endo)plasmic Ca^{2+}-ATPase isoform (SERCA2a) complex is a second determinant. Because each of the new domain Ib mutants contained a normal ratio of pentamer to monomer in SDS-polyacrylamide gel electrophoresis, gain of function must have resulted from mechanisms other than destabilization of pentameric structure. Evidence that domain Ib and domain II mutants act through different sites and different mechanisms was provided by a monomeric double mutant, N30A/I40A, in which the enhanced inhibitory function of each single mutant was additive. Evidence for an alteration in stability of the PLN/SERCA2a heterodimer was obtained in a study of double mutant N27A/N34A in which inhibitory function was regained by combining a gain of function, domain Ib mutation with a loss of function domain II mutation. These results support the proposal that PLN inhibition of SERCA2a involves, first, depolymerization of PLN and, second, the formation of inhibitory interactions between monomeric PLN and SERCA2a.

Phospholamban (PLN) is a 52-amino acid integral membrane protein (1) that interacts with and reversibly inhibits the activity of the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (2). PLN has been predicted to contain three domains. Domain Ia, residues 1–20, contains 6 charged amino acids and has a net positive charge. Phosphorylation of Ser^{16} and Thr^{17}, which alters the net charge of domain Ia, disrupts the inhibitory function of PLN (3, 4). Domain Ia is believed to be at least partly helical, but helicity may not extend beyond Ser^{16} so that domain Ib, residues 21–30, may be relatively unstructured (5). However, studies by Tatulian et al. (6) led to the proposal that it might form a β-sheet structure at the membrane interface. Domain Ib contains a single positive charge in the form of Arg^{25} in most species, but in human, Lys replaces Asn^{27} resulting in 2 positive charges (7). Domain II, residues 31–52, is composed of nonpolar and hydrophobic amino acids, forming a transmembrane helix. In SDS-polyacrylamide gel electrophoresis, PLN mobility corresponds to the mass of a homopentamer, but boiling in SDS reduces the apparent mass to that of a monomer or intermediate oligomers (8).

In a series of papers (9–12), we have coexpressed PLN or PLN mutants with SERCA2a or SERCA2a mutants to define sites of interaction between PLN and SERCA2a. We have used the tools of chimera formation and site-directed mutagenesis to alter function, and we have assayed $K_{ca}$, the Ca^{2+} concentration required for half-maximal activation of Ca^{2+} transport, as a measure of the degree of alteration of the inhibitory association between PLN and SERCA2a. These studies have allowed us to show that the cytoplasmic interaction sites are formed by charged and hydrophobic amino acids in PLN domain Ia (9) and by amino acids Lys-Asp-Asp-Lys-Pro-Val^{402} in SERCA2a (10). We also found that inhibitory interactions occur when only PLN residues 28–52, containing 3 domain Ib residues plus all of the domain II transmembrane residues, are coexpressed with SERCA2a (11). These studies demonstrated that it is the transmembrane amino acids (and possibly a few additional amino acids at the cytoplasm/membrane boundary) that form the inhibitory interaction sites between PLN and SERCA2a. We proposed that inhibitory interactions in the transmembrane sequences of PLN and SERCA2a can be modulated through long range interactions by the noninhibitory cytoplasmic interaction sites and vice versa, so that regulation of SERCA2a function results from interactions among a 4-base circuit composed of 2 interaction sites in PLN and 2 in SERCA2a.

We used alanine-scanning mutagenesis to show that the inhibitory interaction site in the transmembrane sequence of PLN lies on a helical face that must interact with one or more transmembrane helices in SERCA2a, whereas the other helical face is involved in pentamer formation (12). Because the most
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For each mutant, the normal amino acid residue is defined on the left in a single-letter code, its position in the sequence is identified by a number, and the newly introduced amino acid residue is defined on the right in a single-letter code. $K_{Ca}$ is the Ca$^{2+}$ concentration at which half-maximal Ca$^{2+}$ uptake rates were observed. The vertical dashed line on the left represents the $K_{Ca}$ value for SERCA2a expressed in the absence of PLN ($K_{Ca} = 6.59$, designated as 0.0) and the vertical dashed line on the right represents $K_{Ca}$ in the presence of wild type PLN ($K_{Ca} = 6.25$, designated as −0.34). Thus the $\Delta K_{Ca}$ values on the abscissa are negative relative to the $K_{Ca}$ for SERCA2a alone (the apparent affinity of SERCA2a for Ca$^{2+}$ concentration). The Ca$^{2+}$ transport activity expressed in pCa units) from 6.59 to 6.25 (Fig. 1A). The reduction in the apparent affinity of SERCA2a for Ca$^{2+}$ of −0.34 pCa units provided a measure of the inhibitory function of wild type PLN, which was used as a standard for measurement of the gain or loss of inhibitory function by PLN mutants in which each residue in inhibitory mutants of PLN were devoid of pentamer, we deduced that the pentamer represents an inactive reservoir of subunits and that the PLN monomer is the active, inhibitory form. Studies of a single monomeric PLN mutant, L37A, led Cornea et al. (13) and Autry and Jones (14) to a similar view.

In our earlier study of interactions between domain Ib and SERCA2a (9), scanning mutagenesis of domain Ib was incomplete, and none of the mutations that were carried out affected the inhibitory properties of PLN. In the present study, we have completed the alanine-scanning mutagenesis of domain Ib. We show that specific mutations in the C-terminal end of domain Ib also affect the inhibitory properties of PLN. Substitution of Ala for 3 amidated residues, Asn27, Gln29, and Asn30, enhanced PLN inhibition without destablizing the pentameric structure of PLN. These residues are likely to contribute to the stability of the inhibited PLN/SERCA2a heterodimer.

MATERIALS AND METHODS

Mutagenesis, Expression, and Assay—For mutagenesis, a 172-base pair fragment containing the coding sequence for rabbit PLN (bases −6 to 162) (13) was amplified in a recombinant polymerase chain reaction (16) using primers with 5′-add-on sequences containing restriction endonuclease sites for XbaI (5′ end) and SalI (3′ end). The product was subcloned into pBluescript KS+ (Stratagene) after digestion with XbaI and SalI. Mutagenesis with this construct as a template was carried out as described previously (17, 18). Wild type and mutant PLN cDNAs were ligated into the XbaI and SalI sites of the pMT2 expression vector (19) and amplified. Plasmid DNA was purified by adsorption to and elution from Qiagen tip-500 columns. PLN and SERCA2a cDNAs in the pMT2 vector were cotransfected (8 μg of each cDNA per dish) into human embryonic kidney cell line 293 (HEK-293 cells) using the calcium phosphate precipitation method (20). Cells were harvested 48 h after transfection, and microsomes were prepared and assayed for Ca$^{2+}$ transport activity as described previously (9). Data were analyzed as described previously (11).

Immunoblotting—Immunoblotting of SERCA2a and wild type and mutant forms of PLN expressed in HEK-293 cell microsomes was carried out with 10 μg of microsomal proteins. Proteins were solubilized in SDS buffer at room temperature, separated on 12.5% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes as described previously (11), except that PROTRAN nitrocellulose membranes with a pore size of 0.05 microns (Schleicher & Schuell) were used during protein transfer. Membranes were incubated with monoclonal antibody 1D8F6 against SERCA2a (a kind gift from Dr. Kevin P. Campbell) or antibody 1D11 against PLN (a kind gift from Dr. Robert G. Johnson). Antibody binding was detected by chemiluminescence using an ECL Western blotting detection system (Amersham Pharmacia Biotech). Oligomer/monomer ratios were calculated by scanning densitometry of individual lanes in the exposed films of the immunoblots of the gels. Data were averaged from 13 experiments.

RESULTS

Coexpression of PLN with SERCA2a diminished the affinity of SERCA2a for Ca$^{2+}$, shifting apparent $K_{Ca}$ (the Ca$^{2+}$ concentration for half-maximal Ca$^{2+}$ transport activity expressed in pCa units) from 6.59 to 6.25 (Fig. 1A). The reduction in the apparent affinity of SERCA2a for Ca$^{2+}$ of −0.34 pCa units provided a measure of the inhibitory function of wild type PLN, which was used as a standard for measurement of the gain or loss of inhibitory function by PLN mutants in which each residue in
domain Ib was sequenced to alanine (Fig. 1A).

PLN domain Ib mutants P21A, R25A, Q26A, and L28A lost inhibitory function relative to PLN, as indicated by \( \Delta K_{Ca} \) values between 0.19 and 0.25 pCa units. PLN mutants N27A, Q29A, and N30A gained inhibitory function relative to PLN, as indicated by \( \Delta K_{Ca} \) values ranging from -0.62 to -1.08 pCa units. PLN mutants Q22A, Q23A, and A24V had unaltered function, as indicated by \( \Delta K_{Ca} \) values not significantly different from -0.34 pCa units. In addition, PLN domain Ia mutants, A11V and A15V, not previously analyzed, had unaltered function, as indicated by \( \Delta K_{Ca} \) values not significantly different from -0.34 pCa units. Loss or gain of PLN function could not be correlated with different levels of expression (Fig. 2). Moreover, gain of function is not induced by overexpression of PLN under the conditions of our coexpression studies (11).

In further characterization of two of the gain of function mutants that had been characterized earlier (12). We created a double mutant, N30A/I40A, which combined two gain of function mutations, one pentameric and located in domain Ib and one monomeric and located in domain II (Fig. 1C). The \( \Delta K_{Ca} \) for this strongly inhibitory double mutant was -1.08 pCa units over SERCA2a alone or -0.74 pCa units over SERCA2a plus PLN. The \( \Delta K_{Ca} \) for the single monomeric 140A mutant was -0.78 pCa units over SERCA2a alone or -0.44 pCa units over SERCA2a plus PLN. The \( \Delta K_{Ca} \) for the single pentameric N30A mutant was -0.62 pCa units over SERCA2a alone or -0.28 pCa units over SERCA2a plus PLN. Thus the gain of inhibitory function over wild type PLN for the double mutant N30A/I40A (-0.74 pCa units) was equivalent to the additive gain of inhibitory function over wild type PLN for the I40A plus N30A mutations (-0.44 + -0.28 = -0.72 pCa units).

L31A and N34A are pentameric mutants in domain II that are devoid of inhibitory function (12). When these mutations were combined with the strong gain of function pentameric mutation N27A in domain Ib, the double mutant N27A/N34A regained significant inhibitory function (Fig. 1C) and mutant N27A/L31A tended toward gain of inhibitory function. Combination of the weaker gain of function domain Ib mutations, Q29A or N30A, with N31A or N34A, however, did not result in regain of inhibitory function (Fig. 1C).

DISCUSSION

In a series of papers (9–12), we have identified residues in PLN and SERCA2a that are involved in the interactions between these two proteins. In Fig. 1A, we have combined data published earlier (9) with the results of the present study to provide a single map for the results of alanine-scanning mutagenesis of PLN domains Ia and Ib. Fig. 1A, together with Fig. 1A in Ref. 12, provides a complete alanine-scanning mutagenesis map for PLN. These data highlight the fact that mutation to Ala of specific charged (Glu\(^2\), Arg\(^9\), and Arg\(^14\)) and hydrophobic (Val\(^4\), Leu\(^7\), and Ile\(^12\)) residues in domain Ia leads to loss of function. Loss of inhibitory function also occurs with mutation to Ala of Pro\(^{21}\), Arg\(^{25}\), Gln\(^{26}\), and Leu\(^{28}\) in domain Ib and with mutation to Ala of residues Leu\(^{31}\), Asn\(^{34}\), Phe\(^{35}\), Ile\(^{38}\), Leu\(^{42}\), Ile\(^{48}\), Val\(^{49}\), and Leu\(^{52}\) in domain II (12). The results suggest that each of these residues provides an important ligand for the establishment of the circuit of interacting sites that permits PLN to modulate the function of SERCA2a (11).

Fig. 1A also highlights the fact that gain of function accompanied the mutation to Ala of Asn\(^{27}\), Gln\(^{29}\), and Asn\(^{30}\) in domain Ib, whereas Fig. 1A in Ref. 12 illustrates that gain of function accompanies the mutation to Ala of Phe\(^{32}\), Ile\(^{33}\), Leu\(^{37}\), Ile\(^{40}\), Leu\(^{43}\), Ile\(^{47}\), Met\(^{50}\), and Leu\(^{51}\) in domain II. Different explanations are required for the gain of function that follows mutation of specific residues in domain Ib and for the gain of function that follows mutation of specific residues in domain II.

In an earlier study (12), we proposed that the formation of an
inhibited PLN-SERCA2a complex would require two separate steps, the first being the dissociation of the PLN pentamer to form the inhibitory monomeric species and the second being the association of the PLN monomer with SERCA2a. In our hypothesis, gain of function could result from pentamer destabilizing mutations in domain II, which would increase the monomer concentration, and through mass action, increase the concentration of the inhibited complex or through other mutations for example those in domain Ib, which could stabilize the inhibited PLN-SERCA2a complex.

As a first test of the hypothesis, we carried out an indirect measurement of the association between PLN and SERCA2a. Because increased Ca\(^{2+}\) can dissociate the PLN-SERCA2a complex (4), we measured the Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport for each mutant. The fact that higher concentrations of Ca\(^{2+}\) were required to activate SERCA2a, Ca\(^{2+}\) transport was in line with a tighter association between PLN and SERCA2a in domain Ib mutants N27A, Q29A, and N30A.

As a further test of the hypothesis, we combined two strong inhibitory mutations, one from domain Ib and one from domain II. The gain of function for the double mutant N30A/I40A was equal to the sum of the gain of function for the N30A mutant plus the gain of function for the I40A mutant. This observation is in line with our view that these two mutations represent different mechanisms of inhibition, the effects of which are additive.

In a third test of the hypothesis, we also combined mutants from domains II and Ib. The two domain II mutants, L31A and N34A, were devoid of function, presumably because the mutation had diminished their affinity for complementary interacting residues in the transmembrane domain of SERCA2a. Within domain II, loss of function is dominant over gain of function (12). They were combined with the strong gain of function mutants, N27A and N30A, which according to our hypothesis would enhance the affinity between PLN and SERCA2a. In line with our hypothesis, we recovered significant inhibitory function for the double mutant, N27A/N34A, and we observed a trend toward regain of function for the double mutant N27A/L31A. This suggests that the gain in strength of the inhibitory interaction for the mutant N27A was sufficient to overcome the loss in strength of the inhibitory interaction for the mutant N34A. When we combined the N31A or N34A mutations with either of two weaker gain of function mutations, Q29A or N30A, we did not observe regain of inhibitory function in the double mutants, suggesting that the gain in strength of the inhibitory interactions in mutants Q27A and N30A was insufficient to overcome the loss in strength of the inhibitory interactions induced by the mutations L31A or N34A.

The results of analysis of double mutants are fully consistent with the postulate that gain of function domain Ib mutants act through a different mechanism from gain of function domain II mutants. They are also consistent with the view that domain II mutants act through PLN depolymerization, whereas domain Ib mutants act through stabilization of the inhibited PLN-SERCA2a complex.

The question of how the mutation of domain Ib residues leads to gain of inhibitory interaction with SERCA2a was investigated by further mutagenesis of Asn\(^{27}\) and Asn\(^{30}\). The mutant N27D (gain of negative charge) had slight loss of function, the mutant N27K (gain of positive charge and size) had slight gain of function, whereas the mutant N27Q (increased size) had unaffected function. The mutant N30D (gain of negative charge) had unaltered function, whereas the mutant N30Q (increased size) gained function. These results indicate that hydrophilic residues at positions 27 and 30 manifest weaker inhibitory properties than the small hydrophobic residue, Ala. An acidic residue at position 27 exhibited the weakest inhibitory property. The effect of charge in domain Ib seems to be far less dramatic than it is in domain Ia (9).

Overall results, showing rather minor effects over a range of charge and a small increase in size, do not provide a clear picture of the type of interaction between PLN and SERCA2a that might involve Asn\(^{27}\), Gln\(^{29}\), and Asn\(^{30}\). Mutations in domain Ib may increase the affinity of the inhibitory interaction between PLN and SERCA2a through direct interactions with stalk sequences in SERCA2a, or they may occur through the long range transmission of the effects of an altered conformation. Under normal conditions, it is unlikely that PLN/SERCA2a interactions are profoundly influenced by amino acids in this region of domain Ib, especially since Asn\(^{27}\) is not highly conserved, being mutated to Lys in humans (7). In this respect, it is conceivable that the highly inhibitory N27A mutation or the corresponding mutation in humans, K27A if it occurred naturally, could lead to potential cardiomyopathies as predicted earlier for gain of function mutants in domain II (12).

High resolution structures of both PLN and SERCA2a will be required before we can define the specific interactions between PLN and SERCA2a with greater accuracy. In the meantime, the biochemical approach in our laboratory is being directed toward the discovery of the amino acids in SERCA2a that are likely to interact with the loss of function residues in domains Ib and II of PLN that have been identified in this study and in an earlier study (12).

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