

Phospholamban domain I/cytochrome b_5 transmembrane sequence chimeras do not inhibit SERCA2a

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Abstract A series of chimeras between the transmembrane domains of phospholamban (PLN) and cytochrome b_5 were coexpressed with the Ca^{2+} -ATPase of cardiac sarcoplasmic reticulum (SERCA2a). The chimeric molecules were not inhibitory, in line with our view that inhibitory PLN/SERCA2a interactions occur in transmembrane sequences, while cytoplasmic interactions regulate the inhibitory interactions in a four-base circuit.

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1. Introduction

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). In this role, it is a major regulator of the kinetics of cardiac contractility [2]. The protein can be divided into three domains: domain Ia, residues 1–20, is a charged, largely helical, cytoplasmic domain; domain Ib, residues 21–30, is a relatively unstructured sequence, rich in amidated residues; and domain II, residues 31–52, is a transmembrane helix composed of uncharged and hydrophobic amino acids.

Domain Ia contains a site for protein kinase A phosphorylation at Ser¹⁶ and a site for calmodulin kinase II phosphorylation at Thr¹⁷ [3]. Phosphorylation of domain Ia disrupts both the inhibitory effect of PLN on SERCA2a function [4] and the physical interaction between the two molecules [5]. We have used coexpression and site-directed mutagenesis to identify charged and hydrophobic amino acids in PLN domain Ia [6] and charged and hydrophobic amino acids, lying between Lys³⁹⁷ and Val⁴⁰² in the cytoplasmic domain of SERCA2a [7], as the amino acids involved in PLN/SERCA2a cytoplasmic interactions.

An increase in Ca^{2+} concentration also disrupts PLN/SERCA2a interactions [5]. Since the only sites of high affinity Ca^{2+} binding in the PLN/SERCA2a complex lie in SERCA2a transmembrane helices 4, 5 and 6 [8], it is probable that Ca^{2+} binding to M4, M5 and M6 disrupts their inhibitory interactions with PLN domain II. Studies by Sasaki et al. [9] showed that the reconstitution of very high concentrations of soluble

PLN^{1–28} with uninhibited SERCA2a resulted in a decrease in V_{max} , while reconstitution of very high concentrations of insoluble PLN^{28–48} with uninhibited SERCA2a led to a decrease in K_{Ca} . On this basis, Sasaki et al. [9] proposed two sites of inhibitory interaction between PLN and SERCA2a. Further studies in other laboratories have either confirmed [10,11] or failed to confirm [12] the inhibitory role of PLN domain I. Another study [13] disputed the inhibitory role of PLN domain II on the basis that it uncoupled ATP hydrolysis from Ca^{2+} transport.

We coexpressed PLN transmembrane domains with SERCA2a and obtained clear evidence that the transmembrane interaction sites are the inhibitory sites [14]. These observations were strengthened by the use of alanine-scanning mutagenesis to localize the site of transmembrane inhibitory interactions to one face of the PLN transmembrane helix [15]. On this basis, we proposed a model in which the inhibitory interaction sites are located in transmembrane helices and non-inhibitory interactions occur in the cytoplasm. These sites, however, form a four-base circuit, involving both cytoplasmic and transmembrane interaction sites in both PLN and SERCA2a, which are coregulated by either phosphorylation or elevated Ca^{2+} or both [14].

In the continued evaluation of this model, we have created a chimera between PLN cytoplasmic domain I and the transmembrane sequence of cytochrome b_5 [16]. Coexpression of this chimera with SERCA2a does not inhibit SERCA2a function, in line with our proposal that cytoplasmic PLN/SERCA2a interaction sites are not inhibitory.

2. Materials and methods

2.1. Synthesis of chimeric constructs, expression, assay and detection of protein synthesis and phosphorylation

PLN and SERCA2a constructs, their coexpression, assay of their synthesis by immunoblotting, assay of Ca^{2+} transport function by SERCA2a and assay of phosphorylation of PLN have been described previously [14]. The rabbit cytochrome b_5 (cytb5) construct was a kind gift from Dr. David Andrews, McMaster University. PLN^{1–21}-cytb5^{101–134}, PLN^{1–29}-cytb5^{109–134}, and PLN^{1–44}-cytb5^{124–134} were synthesized using a recombinant polymerase chain reaction [17]. Wild type and mutant cDNAs were ligated into the *Xba*I and *Sal*I sites of the pMT2 expression vector [18] prior to expression.

2.2. Immunostaining of transfected cells

Colocalization of SERCA2a and chimeric PLN was evaluated by immunostaining of cells harvested at 48 h after transfection and fixed and permeabilized in 5% acetic acid in ethanol for 5 min on ice. Cells were incubated with rabbit polyclonal antibody C4 against SERCA2a [19] and mouse monoclonal antibody 1D11 against PLN [20] (a kind gift from Dr. Robert Johnson, Merck Research Laboratories), followed by incubation with FITC-labelled anti-rabbit IgG and

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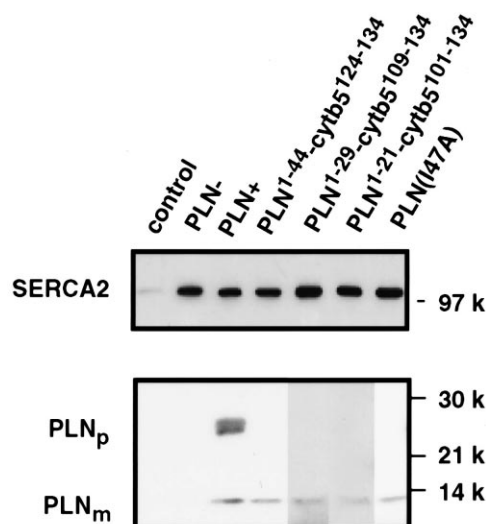


Fig. 1. Immunoblotting of SERCA2a and PLN constructs. Microsomal fractions were isolated from HEK-293 cells and 10 μ g of protein were separated on 12.5% polyacrylamide gels. The gels were divided horizontally at a level corresponding to the mobility of 46 kDa. The upper segment was transferred electrophoretically to a 0.45 μ m nitrocellulose membrane over a 90 min period and the nitrocellulose strip was incubated with anti-SERCA2 monoclonal antibody IID8F6. The lower segment was transferred to 0.05 μ m nitrocellulose membranes for 45 min period and the nitrocellulose strip was incubated with anti-phospholamban antibody 1D11. HRP-conjugated anti-mouse IgG (Promega) was used as the secondary antibody. After addition of the substrates for chemiluminescence, the membranes were exposed to Kodak Biomax M film for 5 s (SERCA2), 1 min (PLN¹⁻²¹-cytb5¹⁰¹⁻¹³⁴ and PLN¹⁻²⁹-cytb5¹⁰⁹⁻¹³⁴) and 15 s for PLN and other PLN constructs. PLN_p and PLN_m refer to pentameric and monomeric forms of PLN, respectively.

TRITC-labelled anti-mouse IgG and examination by confocal microscopy. Excitation wavelengths were 488 nm (FITC) and 568 nm (TRITC) and emission wavelengths were 515–540 nm (FITC) and 590 nm (TRITC).

3. Results

Chimeric mutants PLN¹⁻²¹-cytb5¹⁰¹⁻¹³⁴, PLN¹⁻²⁹-cytb5¹⁰⁹⁻¹³⁴, and PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ were coexpressed with SERCA2a (Fig. 1). While the apparent synthesis of PLN¹⁻²¹-cytb5¹⁰¹⁻¹³⁴ and PLN¹⁻²⁹-cytb5¹⁰⁹⁻¹³⁴ was very low, the synthesis of PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ was approximately equivalent to the level of synthesis routinely obtained with the strongly inhibitory domain II mutant, I47A, described elsewhere [15]. None of the chimeric mutants appeared to be oligomeric in SDS-PAGE.

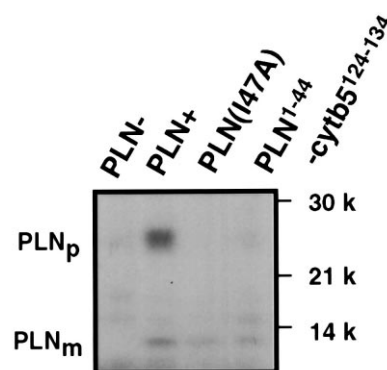


Fig. 2. Phosphorylation of PLN, PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ and PLN(I47A). Microsomes (20 μ g) were phosphorylated with 25 U of the catalytic subunit of cAMP-dependent protein kinase (Sigma) in 30 μ l of 0.15 M KCl, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 20 μ M CaCl₂, 10 mM MgCl₂, 3 mM β -mercaptoethanol, 25 mM NaF and 25 μ M [γ -³²P]ATP for 5 min at room temperature. The phosphorylated microsomes were washed by centrifugation and separated on a 12.5% polyacrylamide gel without boiling. The gel was dried and exposed to Kodak Biomax MR film for 6 h at -70° C with an intensifying screen.

Evaluation of the level of expression of PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ was also carried out by measurement of the amount of ³²P incorporated into PLN or PLN constructs (Fig. 2). The fact that the chimeric PLN was phosphorylated by protein kinase A suggests that domain Ia was properly folded in the chimera and that it was inserted in the membrane in the proper orientation, with the phosphorylation site on the cytoplasmic surface.

Targeting of the chimeric protein to the appropriate intracellular membrane was tested using confocal microscopy of permeabilized and antibody-treated cells to determine whether antibodies raised against SERCA2a and against PLN would be localized to the endoplasmic reticulum and whether the two antibodies would be colocalized. The results shown in Fig. 3 demonstrate that both SERCA2a and the PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ chimera were colocalized in the endoplasmic reticulum.

Data in Table 1 demonstrate that coexpression of SERCA2a with PLN shifts the apparent K_{Ca} (the Ca²⁺ concentration required for half-maximal Ca²⁺ transport, expressed in pCa units) for SERCA2a from 6.59 to 6.25. Coexpression of SERCA2a with mutant I47A reduced apparent K_{Ca} from 6.59 to 6.12. Coexpression of SERCA2a with approximately the same concentration of chimeric mutant PLN¹⁻⁴⁴-cytb5¹²⁴⁻¹³⁴ did not result in any significant change in apparent K_{Ca} .

Table 1
Effects of PLN/cytb5 chimeras on the apparent affinity of SERCA2a for Ca²⁺

Constructs coexpressed with SERCA2a	K_{Ca} (pCa)	<i>n</i>
None	6.59 $\pm$ 0.08	15
PLN	6.25 $\pm$ 0.09*	15
PLN ¹⁻²¹ -cytb5 ¹⁰¹⁻¹³⁴	6.62 $\pm$ 0.04 [†]	5
PLN ¹⁻²⁹ -cytb5 ¹⁰⁹⁻¹³⁴	6.61 $\pm$ 0.05 [†]	5
PLN ¹⁻⁴⁴ -cytb5 ¹²⁴⁻¹³⁴	6.55 $\pm$ 0.12 [†]	4
PLN(I47A)	6.12 $\pm$ 0.11* [†]	8

K_{Ca} is the Ca²⁺ concentration at which half-maximal Ca²⁺ uptake rates were observed, expressed in pCa units. Data are mean values expressed in pCa units $\pm$ S.D. * P < 0.05 vs. none by analysis of variance, followed by the Scheffé F -test. [†] P < 0.05 vs. PLN by analysis of variance, followed by the Scheffé F -test.

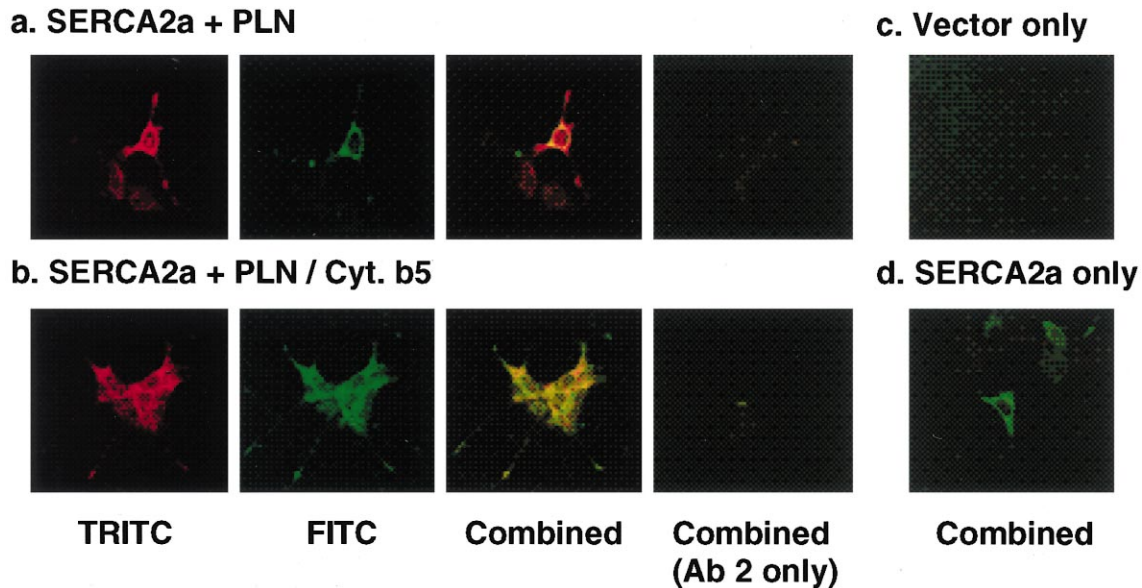


Fig. 3. Colocalization of SERCA2a and PLN^{1–44}-cytb5^{124–134}, coexpressed in HEK-293 cells. Cells were incubated with rabbit polyclonal antibody C4 against SERCA2a [19] and mouse monoclonal antibody 1D11 against PLN [20], followed by incubation with FITC-labelled anti-rabbit IgG and TRITC-labelled anti-mouse IgG and examination by confocal microscopy. a: Transfection with SERCA2a and PLN. b: Transfection with SERCA2a and PLN^{1–44}-cytb5^{124–134}. c: Transfection with pMT2 vector only. d: Transfection with SERCA2a only. In the fourth panels of sections a and b, the first antibody was omitted.

4. Discussion

It is not entirely clear whether cytoplasmic PLN/SERCA2a interactions contribute to the inhibition of SERCA2a function, since publications on this subject are contradictory [9,10,12,21]. In our earlier experiments, interactions between transmembrane helices of PLN and SERCA2a were clearly inhibitory and could account for the major properties of PLN inhibition [14]. Because the cytoplasmic phosphorylation site was missing or inaccessible to protein kinase A in our truncated constructs, reversal of inhibition by phosphorylation of the cytoplasmic domain was not demonstrable. Inhibition could be reversed in our experiments, however, by addition of a Flag antibody to an inhibitory Flag-tagged domain II sequence.

Our attempts to demonstrate inhibitory interactions between PLN domain I and SERCA2a through coexpression of SERCA2a with a chimera composed of PLN domain Ia, domain Ib, part of domain II and part of the transmembrane domain of cytochrome *b*₅ were negative. These negative results, however, are in line with our earlier observations that the cytoplasmic domain of PLN is not required for inhibitory interactions [14] and that even minor alterations in PLN domain II lead to loss of inhibitory function [15]. There are, however, several caveats in this approach. One concern is the level of synthesis that we could obtain. The level of synthesis (Figs. 1 and 2) appears to be low, but is, in fact, underestimated by the transfer and blotting techniques used. The very small size and hydrophobicity of the monomeric PLN and PLN/cytb5 chimeras ensures that they will not be completely captured on nitrocellulose blots. Accordingly, we have used a monomeric mutant, I47A, as a standard for measurement of synthesis. Under conditions where comparable levels of monomeric protein were found adhering to nitrocellulose blots (Fig. 1), I47A was

highly inhibitory, but the PLN/cytb5 chimera was not inhibitory.

The potential caveats of misfolding and improper orientation of the chimera in the membrane have been addressed by demonstration of phosphorylation of the chimera (Fig. 2). The caveat of potential mistargeting has been addressed by studies of the colocalization of the chimeric PLN and SERCA2a presented in Fig. 3.

A final potential caveat is the possibility that expression of a different transmembrane sequence simply prevents the close association of PLN with SERCA2a that might be required for inhibitory cytoplasmic interactions. We cannot rule out this possibility, but we point out that 14 of the 22 residues in the transmembrane sequence were derived from PLN in the PLN^{1–44}-cytb5^{124–134} chimera. While many point mutations in the remaining eight transmembrane amino acids will disrupt PLN/SERCA2a interactions, there is no reason to believe that their alteration would mislocate PLN in its steric relationship to the cytoplasmic interaction site in SERCA2a. Moreover, the fact that PLN domain I has the potential for attachment to and detachment from its binding site on SERCA2a [5] suggests that domain I might have more flexibility than the lipid-bound domain II in approaching its interaction site on SERCA2a and might not be hindered in this interaction by its membrane tether.

The approach of presenting domain I to SERCA2a in different contexts has produced variable results when $V_{\max}$ is measured [9,10,12], but has consistently shown no effect on K_{Ca} . PLN does not affect $V_{\max}$ of SERCA2a, or affects it only marginally, at physiological molar ratios [6,12,21,22]. The effects on $V_{\max}$ of domain I alone were observed only at a high molar ratios of domain I to SERCA2a [9,10]. The modulation of K_{Ca} of SERCA2a by PLN domain I [4,12,13,20–24] or domain I mutants [6] was observed when domain I was connected to an intact and functional domain II. On the other

hand, removal of domain I did not diminish the inhibitory effect on K_{Ca} of the truncated PLN constructs [14]. Thus domain II does not require domain I to exert its inhibitory function, while domain I requires connection to a functional domain II to manifest its regulatory function. These findings led us to propose that the interaction between PLN domain II and SERCA2a transmembrane helices is inhibitory, but that the inhibitory interactions can be modulated by the non-inhibitory interactions which occur between cytoplasmic domains in the two proteins [14,15]. Functional communication between these widely separated domains must occur through long-range coupling, mimicking the long-range transmission of forces between sites of ATP binding in the cytoplasmic domain of SERCA molecules and the Ca^{2+} binding and translocation sites in the transmembrane domain of the same molecules, which results in Ca^{2+} translocation [25]. In the PLN/SERCA2a heterodimer, however, transmission of forces may follow circuits involving both molecules. This study extends our earlier studies by showing that the presentation of domain Ia, domain Ib and part of domain II attached to part of the transmembrane sequence of cytochrome b_5 , under conditions where the chimeric protein is coexpressed with SERCA2a and colocalized, in the proper topological orientation, also fails to affect any alteration in K_{Ca} of SERCA2a.

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