Structural Requirements for Selective Binding of ISC1 to Anionic Phospholipids*

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Yeast ISC1 (Yer019w) encodes inositolphosphoinositol-lipid- phospholipid-phospholipase C and is activated by phosphatidylserine (PS) and cardiolipin (CL) (Sawai, H., Okamoto, Y., Lubert, C., Mao, C., Bielawksa, A., Domae, M., and Hannun, Y. A. (2000) J. Biol. Chem. 275, 39793–39798). In this study, the structural requirements for anionic phospholipid-selective binding of ISC1 were determined using site-directed and deletion mutants. FLAG-tagged Isc1p was activated by PS, CL, and phosphatidylglycerol (PG) in a dose-dependent manner. Using lipid-protein overlay assays, Isc1p interacted specifically and directly with PS/CL/PG. Lipid-protein binding studies of a series of deletion mutants demonstrated that the second transmembrane domain (TMII) and the C terminus were required for PS binding. Moreover, the TMII and the C terminus domains were sufficient to confer PS binding to a heterologous protein, green fluorescence protein. In addition, mutations of positively charged amino acid residues at the C terminus of ISC1 reduced the activating effects of PS, suggesting involvement of these amino acids in interaction with PS/CL/PG and in the activation of the enzyme. Finally, when separate fragments containing the N terminus-TMI and TMII-C terminus were expressed heterologously, enzyme activity was reconstituted, demonstrating that the interaction of the N terminus and the C terminus is required for activity of Isc1p. These results raise the hypothesis that in the presence of PS/CL/PG, the catalytic domain in the N terminus of Isc1p is “pulled” to the membrane to interact with substrate. These studies provide unique insights into the properties of ISC1 and define a novel mechanism for activation of enzymes by lipids cofactors.

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG M2 antibody, anti-FLAG M2 affinity gel, FLAG peptide, and fatty acid-free bovine serum were obtained from Sigma. Goat anti-mouse peroxidase was acquired from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). choline-methyl-1-14C)SM was synthesized as described (15). All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other reagents were purchased from Sigma.

Yeast Strains and Culture Media—The yeast deletion mutant strain JK9–3d/::ISC1 (JSc1 cells) (MATα trpl1 leu2–3 his4 ura3 ade2 rme1::G418 (15)) was used in this study, and other strains were derived from it. Yeast extract and peptone were from Difco. Synthetic minimal medium (S.D.), S.D./Gal, and Ura dropout supplement were purchased from Clontech.

Mutagenesis—Single point mutations were introduced into pYES2/FLAG-ISC1 (15) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A series of deletion mutants were created by a PCR approach using FLAG-ISC1 cDNA as a template. Each 3′-primer contains a termination stop codon with an EcoRI restriction site, whereas each 5′-primer contains a KpnI restriction site. Truncated mutants were made by an overlap extension PCR approach using FLAG-ISC1 cDNA as a template. All oligonucleotides used in this study are listed in Table I. The PCR products and constructs were subsequently sequenced to check that the desired mutations had been intro-
The nomenclature of various recombinant Isc1p proteins, the PCR primers utilized to create the proteins, the fusion tags present, and the regions of Isc1p encoded by the proteins are shown. F and R refer to the forward and reverse PCR primers, respectively. All primer sequences are oriented 5′-3′.

| Name         | PCR primers/region encoded | Fusion tag |
|--------------|----------------------------|------------|
| 169stop      | F: GGTACCATGATGATGATGATAAGACTGCG (5′) | FLAG |
| 377stop      | F: #1                       | FLAG |
| 450stop      | F: #1                       | FLAG |
| ΔN           | F: GGTACCATGATGATGATGATAAGACTGCG (5′) | FLAG |
| ΔP-loop      | F1: #1                      | FLAG |
| R450A        | F: TCCTCTCTTTTGGCGCGTCTGGAATAGGCG (3′) | FLAG |
| R454A        | F: GCCGTCCTGAAAGTGGGAGTTGCAGTCAGTCGT (5′) | FLAG |
| H468A/H469A  | F: AGTGCGGTGATGAGGGCTGCGATTTCAGACCTGCC (5′) | FLAG |
| K477A        | F: #1                       | FLAG |
| R450A/R454A/K477A | F: #4F, F: #5F, R1: #3R, R2: #5R | FLAG |
| H468A/H469A/K477A | F: #1, F: #3F, F: #5F, R1: #3R, R2: #3R | FLAG |
| 422stop      | F: #1                       | FLAG |
| TMII-C       | F: #2                       | FLAG |

The second transmembrane domain and the cytoplasmic domain of Isc1p encoded by the proteins are shown. F and R refer to the forward and reverse PCR primers, respectively. All primer sequences are oriented 5′-3′.

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**TABLE I**

**Identification of the different Isc1p constructs used in this study.**

Protein Determination, SDS-PAGE, and Western Blotting—Samples for gel electrophoresis were combined with reducing 6x SDS sample buffer and separated by SDS-PAGE. For Western blotting, following separation by SDS-PAGE, proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline/0.1% Tween 20 (TBS-T) containing 5% dried milk. Proteins were then identified by incubating antibodies in the presence of primary antibody (anti-FLAG M2 antibody in 5% dried milk/TBS-T for 1 h) and then incubating with secondary antibody (1:2000 dilution of anti-FLAG antibody) in 5% dried milk/TBS-T for 1 h. Secondary antibody was diluted 1:4000 into 5% dried milk/TBS-T and incubated for 1 h. Finally, proteins were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences) with exposure to Biomax MR film (Eastman Kodak Co.).

**Lipid-Protein Overlay Assay**—Lipid-protein overlay assays were performed as described previously (14, 17). Equimolar amounts of the indicated lipids from chloroform stocks were spotted onto Hybond C film (Eastman Kodak Co.).
Purification of the Overexpressed Protein with a FLAG Tag—Lysates of ISC1-overexpressing cells or GFP was prepared as described above. The lysates were loaded onto anti-FLAG-M2 affinity gel (Sigma), washed with Tris-buffered saline (50 mM Tris (pH 7.5) and 150 mM NaCl), and eluted with 150 ng/μl of FLAG peptide.

Assay of ISC1 Activity—The activity of ISC1 was examined as described with modifications (15). Briefly, cell lysates were incubated in 100 μl of buffer containing 100 mM Tris (pH 7.5), 5 mM MgCl₂, 5 mM dithiothreitol, 0.1% Triton X-100, 10 nmol (6.7 mol%) of PS, 10 nmol of Bio-Rad protein assay reagent, with Tris-buffered saline (50 mM Tris (pH 7.5) and 150 mM NaCl), and 0.1% Triton X-100, 10 nmol (6.7 mol%) of PS, 10 nmol of Bio-Rad protein assay reagent, with Tris-buffered saline (50 mM Tris (pH 7.5) and 150 mM NaCl), and

Binding of ISC1 to PS—We have demonstrated previously that ISC1 is stimulated by PS in a dose-dependent manner (15). To demonstrate a physical interaction between ISC1 and anionic phospholipids, binding studies were carried out using the lipid-protein overlay method as described previously (14, 17). By this method, PS and other phospholipids were immobilized on a nitrocellulose membrane, and binding was assessed by incubation with purified FLAG-ISC1 protein or yeast cell lysate from cells overexpressing FLAG-ISC1 followed by immunostaining with anti-FLAG antibody. Fig. 1 demonstrates the binding of ISC1 to PS. As a control for nongenetic binding of protein to lipids, FLAG-tagged GFPuv was used (Fig. 1, a and b). The binding of purified FLAG-ISC1 to PS was very prominent whereas that of FLAG-GFPuv was hardly detectable (Fig. 1c). Fig. 1c also demonstrates that cell lysates from yeast cells overexpressing FLAG-ISC1 interacted similarly with PS. Therefore, yeast cell lysates were used in subsequent studies. These results suggested that ISC1 may harbor a PS-binding domain.
Binding of Isc1p to Anionic Phospholipids

To evaluate whether other phospholipids are able to interact with Isc1p, the activity of Isc1p using various concentrations of different phospholipids was determined. As shown in Fig. 2a, FLAG-Isc1p was stimulated by PS, CL, PG, phosphatidylinositol, and phosphatidic acid, but not phosphatidylcholine or phosphatidylethanolamine, suggesting that anionic phospholipids activate Isc1p. Fig. 2b shows that using yeast cell lysate from FLAG-ISC1-overexpressing cells, FLAG-Isc1p bound specifically to the anionic phospholipids PS, CL, or PG. FLAG-Isc1p also interacted, but weakly, with phosphatidylinositol or phosphatidic acid and did not bind phosphatidylcholine or phosphatidylethanolamine. These results support the kinetic data (Fig. 2a) and suggest that Isc1p can interact with anionic phospholipids with a clear preference for PG, PS, and CL. Therefore, PS was used in subsequent studies.

The Transmembrane Domains and the C Terminus of Isc1p Are Required for PS Binding

To determine whether there is a discrete PS-binding domain in Isc1p, the activity of Isc1p using various concentrations of different phospholipids was determined. As shown in Fig. 2a, FLAG-Isc1p was stimulated by PS, CL, PG, phosphatidylinositol, and phosphatidic acid, but not phosphatidylcholine or phosphatidylethanolamine, suggesting that anionic phospholipids activate Isc1p. Fig. 2b shows that using yeast cell lysate from FLAG-ISC1-overexpressing cells, FLAG-Isc1p bound specifically to the anionic phospholipids PS, CL, or PG. FLAG-Isc1p also interacted, but weakly, with phosphatidylinositol or phosphatidic acid and did not bind phosphatidylcholine or phosphatidylethanolamine. These results support the kinetic data (Fig. 2a) and suggest that Isc1p can interact with anionic phospholipids with a clear preference for PG, PS, and CL. Therefore, PS was used in subsequent studies.

**Fig. 3.** Binding of deletion mutants of Isc1p to PS. a, schematic diagram of deletion mutants of Isc1p. The various ISC1 constructs used in this study are indicated. All proteins were fused to FLAG at the N terminus. b, immunoblot analysis of deletion mutants of Isc1p with anti-FLAG antibody. c, lipid-protein overlay assay showing the various ISC1 constructs binding to immobilized lipids. Equimolar amounts of lipids were immobilized on nitrocellulose membranes and probed with yeast cell lysates. Lipid-protein binding was identified by immunostaining with an anti-FLAG monoclonal antibody. The results are representative experiments of at least three independent experiments. d, activity of deletion mutants of Isc1p at various concentrations of PS. The results are averages of duplicate experiments. Similar results were obtained in two separate experiments.

**Fig. 4.** Binding of GFP fusion proteins to PS. a, schematic diagram of GFP-fusion proteins. The various ISC1 constructs used in this study are indicated. All proteins were fused to FLAG at the N-terminus. The circle represents FLAG, and the boxes represent the predicted TM. b, immunoblot analysis of GFP fusion proteins with anti-FLAG antibody. c, lipid-protein overlay assay showing the various ISC1 constructs binding to immobilized lipids. Equimolar amounts of lipids were immobilized on nitrocellulose membranes and probed with yeast cell lysates. Lipid-protein binding was identified by immunostaining with an anti-FLAG monoclonal antibody. The results are representative experiments of at least three independent experiments.
deletion mutant (162–169) also bound PS with similar intensity to wild-type protein. These results suggest that the TMs and the C terminus, but not the N terminus or the P-loop, are required for anionic phospholipid-selective binding.

**Fig. 3** indicates the effects of these mutants on activity of Isc1p. The 377-stop, 450-stop, or P-loop truncation mutants lost activity completely. Noticeably the P-loop deletion lost activity but not PS binding. These results show that although the activity of Isc1p requires multiple domains, the interaction with PS (activation and binding) is determined primarily by the TMs and the C terminus.

**The TMII-C Terminus Domain Is Sufficient for PS Binding**—Next, to determine whether there is a domain in Isc1p sufficient to bind PS, a series of FLAG-tagged GFP fusion proteins were constructed and examined. These fusion proteins, consisting of different fragments of ISC1 attached to FLAG-tagged GFP, were expressed in ΔISC1 cells, and a schematic diagram of the constructs is shown in Fig. 4a. Fig. 4b shows a representative Western blot analysis of the GFP fusion proteins and the level of expression of all GFP fusion proteins was found to be consistently higher than the wild-type. The results of lipid-protein overlay assays with GFP fusion proteins are shown in Fig. 4c. Modest binding to PS was observed for FLAG-GFP, which did not show significant binding. On the other hand, the binding of FLAG-GFP-TMII-C terminus (TMII and C terminus fragment of ISC1) to PS was robust, although somewhat weaker compared with FLAG-Isc1p. These results suggest that the anionic phospholipid-selective-binding domain is localized primarily to TMII and the C terminus.
## Activation of Isc1p by PS/CL/PG

Positively Charged Amino Acid Residues Regulate the Interaction with PS—As the above results defined a minimum domain that was necessary and sufficient for PS binding, we searched the database for possible similar motifs. As shown in Fig. 5a, ISC1 appeared to contain a motif first identified in the classical PKCs and shown to be present in PS decarboxylases, and in PLA1, which acts specifically on PS as a substrate (20). Interestingly, this motif was not discovered when the whole sequence of ISC1 was searched but only with the TMI-C terminus domain.

Moreover, this domain contains a number of conserved positively charged amino acids that are therefore candidates for binding the negatively charged PS (21). To test the role of these positively charged amino acid residues in the regulation of ISC1 by PS, we investigated the effects of mutating these amino acids on PS regulation of Isc1p using site-directed mutagenesis (Fig. 5b). Fig. 5c shows a representative Western blot analysis of the C terminus mutants, and the level of expression of mutants was found to be similar. Next, the effects of these positively charged amino acid residues on PS regulation of Isc1p were investigated using kinetic analysis of activation by PS. It should be noted that overlay assays proved inadequate to distinguish quantitative effects on binding and therefore was not utilized in these studies. Fig. 5d shows activity of Isc1p at various concentrations of PS. A dramatic decrease of activity was observed for R454A compared with FLAG-Isc1p. On the other hand, a significant but less pronounced decrease of activity was observed for R450A, H468A/H469A, and K477A compared with FLAG-Isc1p. The activity of H468A/H469A/K477A was similar to H468A/H469A, but R450A/R454A/K477A abolished the activity. The activity of H468A/H469A, K477A, or H468A/H469A/K477A was S-shaped, and the Hill coefficient of activation of these mutants by PS was similar to FLAG-Isc1p (−4). The activation of R450A or R454A by PS was decreased significantly. The Hill coefficient of R450A and R454A were 3.9 and 2.3, respectively. These results suggested that the cooperativity with PS of R454A decreased. These results suggested that Arg-450 and Arg-454 are the primary amino acids involved in the activating effects of PS/CL/PG.

Interaction of the N Terminus and the C Terminus—The above results with the P-loop deletion indicated that the N terminus is required for the activity of Isc1p but not PS binding (Fig. 3), suggesting that the amino “domain” of the enzyme harbors the catalytic site. On the other hand, the above results identify a carboxyl domain that is required for interaction with PS/CL/PG and for activation of the enzyme by these lipids. These results suggested the hypothesis that the function of the carboxyl domain is perhaps to interact with the amino domain, such that when the carboxyl domain binds PS/CL/PG, it is then able to position the catalytic domain to access substrates. Therefore, to examine whether the N- and the C-domains interact, we expressed N terminus-TMI and TMII-C terminus in separate plasmids in ΔISC1 cells. All deletion mutant constructs are shown in Fig. 6a. Fig. 6b shows a representative Western blot analysis of the deletion mutants, and the expected bands of all deletion mutants were observed. When 422-stop and FLAG-GFP-TMII-C terminus were expressed together, both bands were observed. Expression of 422-stop (N terminus-TMI), FLAG-TMII-C terminus, or FLAG-GFP-TMII-C terminus separately did not demonstrate any enzyme activity. On the other hand, when 422-stop and FLAG-TMII-C terminus or FLAG-GFP-TMII-C terminus were expressed in combination, the activity was reconstituted, suggesting that the interaction of the N terminus and the C terminus is required for activity of Isc1p.

**DISCUSSION**

The results of this study demonstrated that Isc1p is capable of interacting with anionic phospholipids, especially PS, CL, and PG. The results define a discrete domain in Isc1p composed of the second transmembrane domain and the carboxyl terminus that functions as anionic phospholipid-selective binding domain. Our studies show that this domain is both necessary and sufficient for binding of PS and for imparting PS-dependent stimulation of activity. The results also pinpoint two major cationic amino acids in this domain that are necessary for the cooperative activation of Isc1p by PS. Finally, the results suggest a mechanism by which the anionic phospholipid-selective binding carboxyl domain activates the enzyme by interacting with the catalytic domain.
Recently, the motif FXFXLXXXXXR found in the C2 domain of classical PKCs, in PS decarboxylases, and in PS-PLA1 was defined based on the ability of an anti-idiotypic antibody raised against the combining site of a PS-specific antibody to interact with PKC (20). Interestingly, residues 446–454 (PLFGRRSEIR) found in the membrane-proximal C terminus of Isc1p are similar to this putative PS-binding motif. However, Johnson et al. (21) reported that this motif is not involved in regulation of PKCβII by lipids using site-directed mutagenesis. In addition, Verdaguer et al. (22) showed that PS binding of PKC relied upon completely different residues. Moreover, the situation with PKC is more complicated by the fact that both the C1 and C2 domain interact with anionic phospholipids but perhaps with different specificities (23, 24). Taken together, our observations suggest that this motif is essential for interaction with PS/CL/PG and not only PS. Thus, the specificity of the interaction of the other proteins that contain this motif with anionic phospholipid should be examined and determined.

Moreover, it has been shown that positively charged amino acid residue(s) play significant roles in some cases of protein-anionic phospholipids interactions. A number of positively charged amino acid residues of DnaA have been implicated by mutagenesis as being involved in binding to anionic phospholipids such as CL (25). Five positively charged amino acid residues (Arg-450, Arg-454, His-468, His-469, and Lys-477) are found in the C terminus of Isc1p. Our site-directed mutagenesis studies indicated that the co-substitution of Arg-450 and Arg-454 with alanine results in significant loss of PS activation of Isc1p and in complete loss of cooperativity of interaction (Fig. 5), suggesting that these two positively charged amino acid residues are critical for the activating effects of PS/CL/PG. Although we did not evaluate the role of the amino-terminal hydrophobic residues (Phe-446 and Phe-448), these Phe residues or other hydrophobic amino acid residues within the TMII may be involved in recognizing the hydrophobic tail of PS/CL/PG, because the TMII is necessary for the binding of Isc1p to PS (Fig. 4).

The results from this study also demonstrated an essential role for the P-loop-like domain in the amino terminus in catalysis but not in binding to PS. Together with the results showing that the C terminus is required for both binding of PS and activation by PS (Fig. 3), we wondered whether the two domains of the enzyme interact. Thus, when the two separate fragments N terminus-TMII and TMII-C terminus were co-expressed, activity was reconstituted (Fig. 6), suggesting that the interaction of the N terminus and the C terminus is required for activity of Isc1p. Interestingly, a cluster of negatively charged amino acid residues is found in the C terminus downstream to Arg-450, Arg-454 (residues 458–467). On the other hand, a cluster of positively charged amino acid residues is found downstream to the catalytic domain in the N terminus (residues 382–398). Therefore, it is possible that these regions interact via electrostatic interactions. These mechanisms are under investigation.

Fig. 7 shows a proposed molecular mechanism for how PS/CL/PG stimulates Isc1p activity. We propose that in the presence of PS/CL/PG, the TMII and the proximal C terminus (anionic phospholipid-selective binding domain) associates with membranes via tethering to anionic phospholipids. As a consequence of this tethering and the interaction of the amino and carboxyl domains of the enzyme, the catalytic domain in the N terminus of Isc1p is pulled to the membrane to interact with lipid substrates. One possible prediction of this model is that colocalization of Isc1p and PS/CL/PG might be critical for the activation of Isc1p.

In conclusion, these results define a novel domain composed of one transmembrane domain (TMII) and the C terminus that is required for anionic phospholipid-selective binding. This domain contains a conserved motif that is required for PS/CL/PG binding with a critical role for positively charged amino acids that cooperatively recognize PS/CL/PG. In addition, we demonstrate that the N terminus and the C terminus interact, and that this interaction plays a critical role in catalysis by Isc1p. These studies provide unique insights into the properties of ISC1 and define a novel mechanism for activation of enzymes by lipid cofactors.

Acknowledgments—We thank Dr. Korey R. Johnson and Kevin P. Becker for advice about the mutagenesis of Isc1p, Jeffrey A. Jones for advice on the lipid-protein overlay assay, Dr. Alicia Bielawska for giving [choline-methyl-3H]SM, and Dr. Maurizio Del Poeta, Dr. Chiara Luberto, and Dr. Cungui Mao for advice on yeast studies.

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J. Biol. Chem. 2002, 277:46470-46477.
doi: 10.1074/jbc.M207779200 originally published online September 18, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207779200

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