Functional Analysis of *ISC1* by Site-Directed Mutagenesis†

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ABSTRACT: We previously reported that the yeast *Saccharomyces cerevisiae ISC1* gene (Yer019w), which has homology to the bacterial sphingomyelinase gene, encodes inositol phosphosphingolipids−phospholipase C, Isc1p [Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domae, M., and Hannun, Y. A. (2000) *J. Biol. Chem. 275*, 39793−39798]. The present study was conducted to determine specific domains in Isc1p required for catalysis. Several amino acid residues are conserved from bacterial sphingomyelinase to mammalian sphingomyelinase and are also found in *ISC1*. Individual mutation of the conserved E100, N233, and H334 resulted in complete loss of Isc1p activity, suggesting an essential role in catalysis for these amino acid residues. Isc1p also contains a domain (from G162 to S169) with homology to P-loop domains, found in nucleotide-binding proteins. In addition, two amino acid residues from this domain, D163 and K168, are conserved from bacterial to mammalian sphingomyelinases in this "P-loop-like domain". G162, D163, G167, K168, and S169 were replaced individually with alanine using site-directed mutagenesis. D163A and K168A lost activity completely. Mutations in the other three positions rendered enzyme versions with much reduced but detectable activity. The V_{max} values for G162A, G167A, and S169A were reduced, compared with wild type, but the K_m values for G162A, G167A, and S169A were similar to that of wild type, indicating that the substrate binding efficiency was not greatly altered in these mutants and that the P-loop-like domain of *ISC1* might be essential in catalysis of Isc1p. Furthermore, the Mg^{2+} K_a constants for G162A, G167, and S169A were higher than that for wild type, suggesting that this P-loop-like domain may be involved in Mg^{2+} binding. Although cell lysates from yeast cells overexpressing all mutants similarly bound to phosphatidylserine (PS), an anionic lipid activator of Isc1p, G162A and G167A required 13.3 mol % PS to achieve maximum activity compared to 6.7 mol % for the wild-type enzyme, suggesting that PS might play a role in optimal catalytic efficiency of Isc1p via this P-loop-like domain. This study provides novel insight into a new domain found in Isc1p and related enzymes.

Recently, two human neutral sphingomyelinases (nSMase1 and nSMase2) have been cloned on the basis of sequence similarity to bacterial sphingomyelinase (*1*, *2*). In addition, enzymes from other eukaryotes including *Saccharomyces cerevisiae* (*ISC1*), *Schizosaccharomyces pombe* (*CSS1*), *Caenorhabditis elegans*, and *Drosophila melanogaster* contain genes that display sequence homology (*3*−*5*). We have reported that *ISC1* (Yer019w) encodes inositol phosphosphingolipid−phospholipase C (Isc1p) and also exhibits neutral sphingomyelinase activity against exogenous sphingomyelin as a substrate (*3*). The three-dimensional structure of the *Bacillus cereus* enzyme has been modeled using the structure determined for DNase I (*6*), which shows some sequence similarities. On the basis of this prediction and supporting experimental evidence, the function of several residues in the *B. cereus* enzyme has been suggested (*6*). For example, G53 is likely to function as a metal ion binding, D126 as a substrate recognition, and H296 as a general base in the catalytic reaction (*6*). On the other hand, D195 and N197 have been suggested to interact with the phosphate group of sphingomyelin (*6*). These amino acid residues are conserved from *B. cereus* to eukaryotes, including *ISC1* (see Figure 1).

Compared with amino acid sequences of other sphingomyelinases, *ISC1* stands out as containing a domain with high homology to P-loop domains, found in nucleotide-binding proteins (*7*). The primary structure of the P-loop domain typically consists of a glycine-rich region followed by a conserved lysine and a serine/threonine (*8*). This P-loop-like domain is localized in the catalytic region of *ISC1*, and closer inspection reveals that all members of the extended family of sphingomyelinases show similarities in this domain with two amino acid residues, D163 and K168, being conserved from bacteria to mammals. However, the role of this P-loop-like domain in Isc1p activity remains unknown.

This study aimed at examining the functional role of conserved amino acid residues and the P-loop-like domain of *ISC1*. To this end, we introduced mutations into the conserved amino acid residues of the P-loop-like domain by site-directed mutagenesis. The results delineate several key conserved amino acid residues, and they demonstrate a role for the P-loop-like domain of *ISC1* in catalysis by Isc1p.

EXPERIMENTAL PROCEDURES

Materials. Anti-FLAG M2 antibody and anti-FLAG M2 affinity gel were obtained from Sigma. Goat anti-mouse

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Table 1: List of the Different Isc1p Constructs Used in This Study[a]

name	PCR primers	fusion tag
E100Q	F: GTGATTGCCTTACAACAAATCTGGTGTGTGG R: CCACACACCAGATTTGTTGTAAGGCAATCAC full-length Isc1p with point mutation (E100Q)	FLAG
N233H	F: GTGGTGGGTGACTTACACTCCAGACCGGGCTCA R: TGAGCCCGGTCTGGAGTGTAAGTCACCCACCAC full-length Isc1p with point mutation (N233H)	FLAG
H334N	F: TGCACTGTCTCTGACAATTTTGCATACTCATGC R: GCATGAGTATGCAAAATTGTCAGAGACACTGCA full-length Isc1p with point mutation (H334N)	FLAG
G162A	F: GAGTGCGGTGTTCCGTGCCGACTGGTACGTAGGG R: CCCTACGTACCAGTCGGCACGGAACACCGCACTC full-length Isc1p with point mutation (G162A)	FLAG
D163A	F: GCGGTGTTCCGTGGCGCCTGGTACGTAGGGAAA R: TTTCCCTACGTACCAGGCGCCACGGAACACCGC full-length Isc1p with point mutation (D163A)	FLAG
G167A	F: GGCGACTGGTACGTAGCGAAATCTATAGCAATC R: GATTGCTATAGATTTCGCTACGTACCAGTCGCC full-length Isc1p with point mutation (G167A)	FLAG
K168A	F: CGACTGGTACGTAGGTGCCTCTATAGCAATCACC R: GGTGATTGCTATAGAGGCACCTACGTACCAGTCG full-length Isc1p with point mutation (K168A)	FLAG
S169A	F: CTGGTACGTAGGGAAAGCTATAGCAATCACCG R: CGGTGATTGCTATAGCTTTCCCTACGTACCAG full-length Isc1p with point mutation (S169A)	FLAG

[a] The nomenclature of various recombinant Isc1p proteins, the PCR primers utilized to create the proteins, and the fusion tags present are shown. F and R refer to the forward and reverse PCR primers, respectively. All primers sequences are oriented 5′ to 3′.

peroxidase was acquired from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). [choline-*methyl*-^{14}C]-SM[1] was synthesized as described (*3*). 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 JK9-3dα/Δ*ISC1* (Δ*ISC1* cells) (MATα trp1 leu2-3 his4 ura3 ade2 rme1 *ISC1::G418*) (*3*) was used in this study, and other strains were derived from it. Yeast extract and peptone were from Difco. Synthetic minimal medium (SD), SD/Gal, and Ura dropout supplement were purchased from Clontech.

Mutagenesis. Single point mutations were introduced into pYES2/FLAG-*ISC1* (*3*) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All oligonucleotides used in this study are listed in Table 1. All constructs were subsequently sequenced to verify that the desired mutations had been introduced into the sequence. All of the constructs were respectively introduced into Δ*ISC1* cells as described (*3*), and gene expression was induced by incubating cells in SC-Ura medium plus 2% galactose.

Protein Determination, SDS−PAGE, and Western Blotting. Samples for gel electrophoresis were combined with reducing 6 × 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 with a 1:2000 dilution of anti-FLAG M2 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 (*9*−*11*). Equimolar amounts of the indicated lipids from chloroform stocks were spotted onto Hybond C extra nitrocellulose membrane (Amersham Biosciences). The membranes were allowed to dry under vacuum for 1 h and were then wetted by floating on purified water. The membranes were equilibrated in TBS-T for 5 min, followed by blocking with 3% fatty acid-free bovine serum albumin/TBS-T (blocking reagent) for 1 h at room temperature. Yeast cell lysate was diluted into blocking reagent to a final concentration of 2 μg/mL. The membranes were then incubated in the presence of the cell lysate overnight at 4 °C on a rocking platform. The following day the membranes were washed six times for 5 min with TBS-T. All subsequent steps were carried out at room temperature. Protein was detected by incubation with a 1:2000 dilution of anti-FLAG M2 antibody in 5% dried milk/TBS-T for 1 h. This was followed by a second wash step of six times for 5 min with TBS-T. Secondary antibody was diluted 1:4000 into 5% dried milk/TBS-T and incubated for 1 h. This was followed by a final wash step of 12 times for 5 min with TBS-T. Finally, the protein was visualized using ECL with exposure to Biomax MR film.

Preparation of Lysates of Yeast Cells. Yeast cells were suspended in buffer containing 25 mM Tris (pH 7.4), 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 4 μg/mL each chymostatin, leupeptin, antipain, and pepstatin A. Cells were disrupted with glass beads as described (*3*). Glass beads and cell debris were removed by centrifugation at 2500*g* for 10 min twice. Protein concentration was determined using the Bio-Rad protein assay reagent.

Assay of Isc1p Activity. The activity of Isc1p was examined as described with modifications using Triton X-100/lipid mixed micelles (*3*, *12*, *13*) which are known to maintain a

[1] Abbreviations: PS, phosphatidylserine; SM, sphingomyelin; TBS-T, Tris-buffered saline/0.1% Tween-20; ECL, enhanced chemiluminescence.

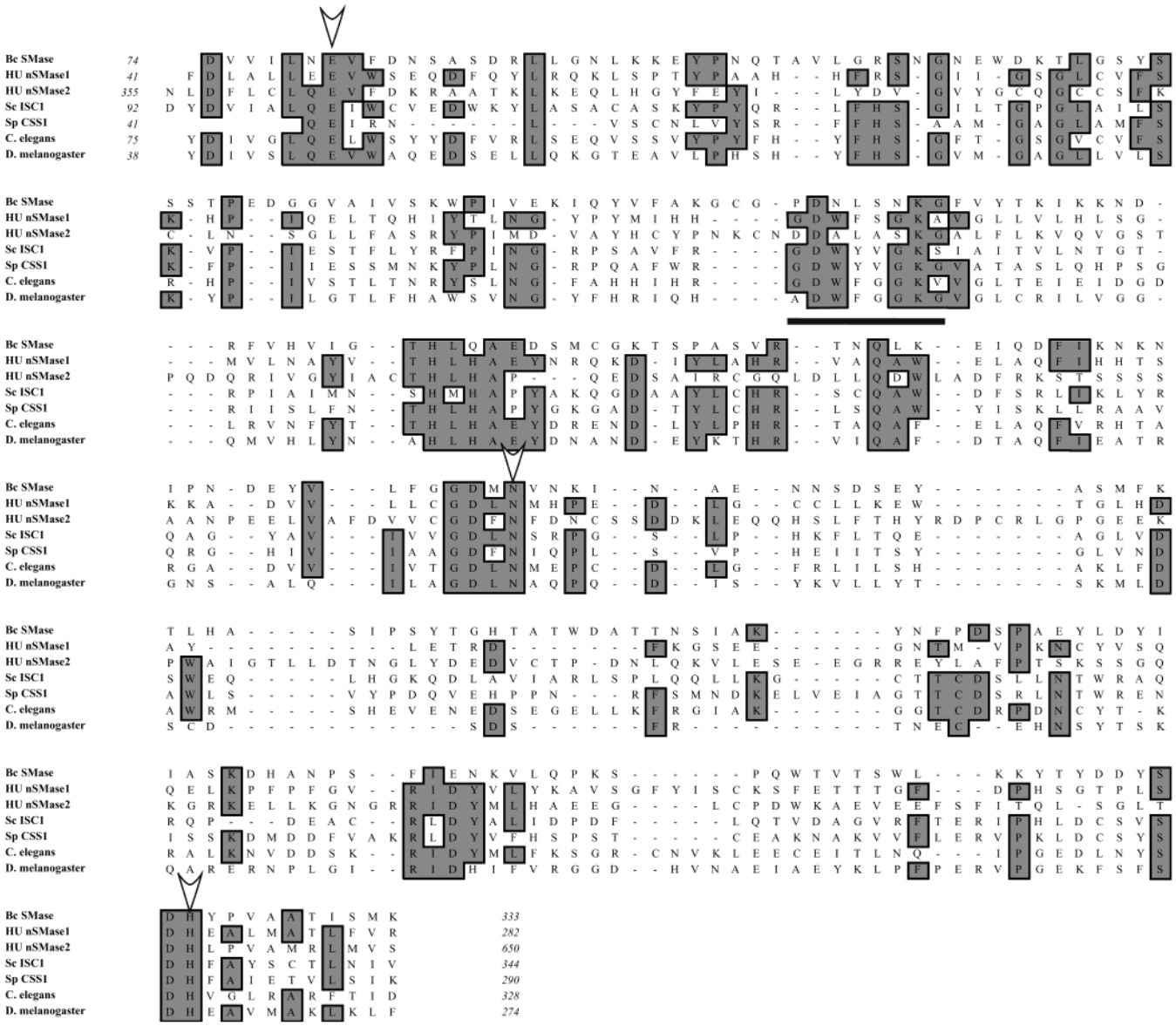

FIGURE 1: Alignment of amino acid sequences with similarity to bacterial SMase from different species. The following sequences (with accession numbers in parentheses) were used for the alignments: *B. cereus* (CAA31333), *S. cerevisiae ISC1* (CAB39367), *S. pombe CSS1* (CAB39367), human nSMase1 (NP003071), human nSMase2 (NP061137), *C. elegans* (CAB04885.1), and *D. melanogaster* (AAF47741.1). Highly conserved regions are shaded and boxed. E100, N233, and H334 are indicated by arrows. P-loop-like domains are underlined.

homogeneous composition and size (of approximately 140 detergent molecules/micelle). Briefly, cell lysates were incubated in 100 μL of buffer containing 100 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1% Triton X-100, 10 nmol (6.7 mol %) of PS, 10 nmol (6.7 mol %) of unlabeled SM, and 100000 dpm of [choline-*methyl*-^{14}C]SM at 30 °C for 30 min. After the incubation, 1.0 mL of chloroform, 0.5 mL of methanol, and 0.2 mL of water were added according to the method of Folch et al. (*14*), and the radioactivity in a portion (400 μL) of the upper phase was measured by liquid scintillation counting.

RESULTS

Functional Analysis of ISC1 by Site-Directed Mutagenesis of Potential Catalytic Amino Acid Residues. Figure 1 shows the comparison of amino acid sequences of sphingomyelinases from *Bacillus cereus* and various species. Recently, the human (nSMase1 and nSMase2) and *S. cerevisiae* (*ISC1*) enzymes have been identified on the basis of sequence similarity to bacterial sphingomyelinase (*1*−*3*). In addition, other eukaryotes including *S. pombe* (*CSS1*), *C. elegans*, and *D. melanogaster* have enzymes with homologous sequences (Figure 1) (*4*, *5*). The three-dimensional structure of the *B. cereus* enzyme has been modeled using the structure determined for DNase I that shows some sequence similarities (*6*). On the basis of this prediction and supporting experimental evidence, the function of several residues in the *B. cereus* enzyme has been suggested (*6*). For example, G53 is likely to function as a metal ion binding, D126 as a substrate recognition, and H296 as a general base in the catalytic reaction (*6*). On the other hand, D195 and D197 were suggested to interact with the phosphate group of sphingomyelin (*6*) (Figure 1). *ISC1* may also share a similar catalytic mechanism with bacterial sphingomyelinase since E100, D234, and H334 of *ISC1* are probably homologous to the critical residues implicated in catalysis in the bacterial sphingomyelinase (Figure 1). In fact, it has been demonstrated that Isc1p exhibits sphingomyelinase activity (*3*).

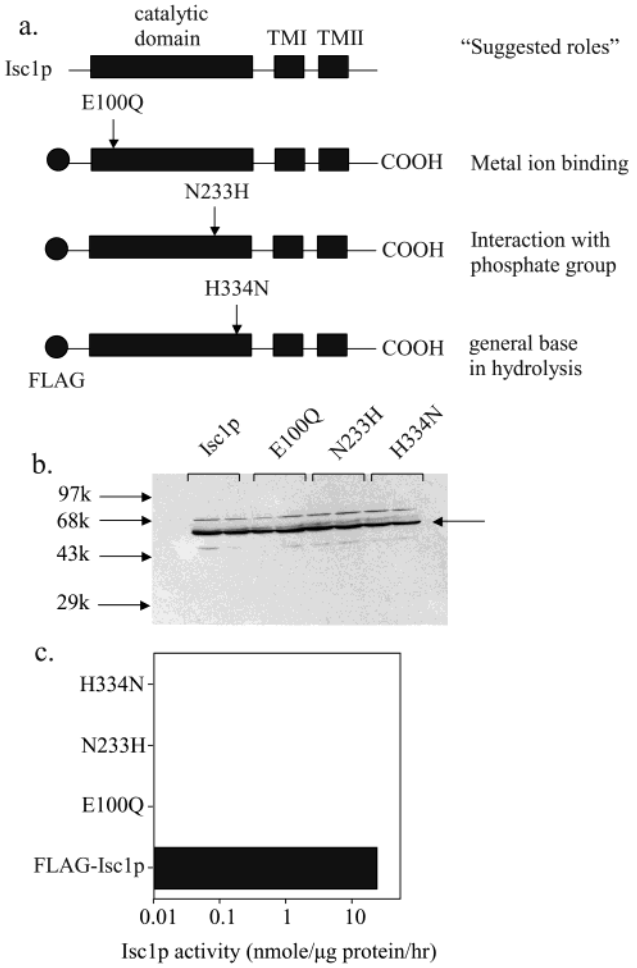

FIGURE 2: Effects of mutations in E100, D234, and H334 residues of Isc1p on activity. (a) Schematic diagram of site-directed mutants of Isc1p. All proteins were fused to FLAG at the N-terminus. The circle represents FLAG, and the boxes represent the putative transmembrane domains (TMs). (b) Immunoblot analysis of site-directed mutants of Isc1p was performed with anti-FLAG antibody. The two lanes correspond to protein extracts from two colonies, the product of individual yeast transformations. (c) Effects of mutations in E100, D234, and H334 of Isc1p on Isc1p activity. Isc1p activity was measured using SM as substrate as described in Experimental Procedures. The results are averages of triplicate experiments. Similar results were obtained in three different experiments.

To test the putative functions of these amino acid residues of *ISC1*, we investigated the effects of mutating these amino acids on activity of Isc1p using site-directed mutagenesis (Figure 2a). All of the constructs were individually introduced into Δ*ISC1* cells, and gene expression was induced by incubating cells in SC-Ura medium plus 2% galactose. Since untransformed Δ*ISC1* cells present no sphingomyelinase activity, this model allowed us to determine changes in activity specific to Isc1p. We also noted that the activity of the partially purified protein from Isc1p overexpressing cells was unstable as described previously (*3*), with loss of more than 90% of activity upon affinity purification. Also, some of the mutants were extremely labile, and therefore for these reasons cell lysates were used in this study for the effects of mutants on Isc1p activity. Moreover, there are conceptual reasons to favor studying transmembrane proteins in their natural membrane environment (especially for labile mutants) as this minimizes secondary changes in structure imparted by solubilization and exposure of hydrophobic regions. Figure 2b shows a representative Western blot analysis of the expressed protein, and FLAG-tagged ~55 kDa bands were detected in all lysates. The level of expression of mutants was found to be similar to that of wild type. Figure 2c indicates the effects of these mutants on activity of Isc1p. The sphingomyelinase enzymatic activity was completely abrogated in the three different mutants. These results provide evidence for the essential function of these three residues, E100, D234, and H334, that are located fairly apart from each other in the primary sequence of *ISC1*, supporting the analogy of the active center of *ISC1* to that of bacterial sphingomyelinase.

Functional Analysis of ISC1 by Site-Directed Mutagenesis of the P-Loop-like Domain. Inspection of the sequence of Isc1p shows that it contains a domain, between G162 and S169, with homology to the P-loop motif found in nucleotide-binding proteins (Figure 1) (*8*). Figure 3a shows the comparison of amino acid sequences of this P-loop-like domain of *ISC1* with other sphingomyelinases from bacteria to mammals. Interestingly, D163 and K168 within this domain are conserved among all sphingomyelinases from bacteria to mammals (Figure 3a). Previous reports suggested that D126 in bacterial sphingomyelinase, which may correspond to D163, is thought to be close to the active site and is related to the catalytic activity and substrate binding (*15*). Although many ATP- or GTP-binding proteins contain this motif, not all proteins that contain this motif bind nucleotides. In activity studies, none of the nucleotides, ATP, ADP, GTP, or GDP, affected Isc1p activity (data not shown).

To further examine the role of this P-loop-like domain of *ISC1* on activity of Isc1p, G162, D163, G167, K168, and S169 (Figure 3b) were replaced individually with alanine using site-directed mutagenesis, and the effects of mutating these amino acids on activity of Isc1p were investigated. All of the constructs were introduced individually into Δ*ISC1* cells, and gene expression was induced by incubating cells in SC-Ura medium plus 2% galactose. To examine the effects of these mutants on Isc1p activity, the cell lysates from yeast cells overexpressing wild type and mutants were used. Figure 3c shows a representative Western blot analysis of these mutants. The overexpressed protein with the FLAG tag was detected at ~55 kDa, and the level of expression of mutants was found to be comparable. Figure 3d shows that D163A and K168A lost activity completely, suggesting that these two conserved amino acids of the P-loop-like domain are essential for catalysis by Isc1p. Mutations in the other three positions rendered enzyme versions with activity much lower than that of the wild-type enzyme but still detectable. Experiments to determine the kinetic parameters for these enzymes were performed and analyzed by Sigma Plot under a Michaelis−Menten model. The V_{max} (nanomoles of choline phosphate liberated per microgram of protein per hour) values for wild type, G162A, G167A, and S169A were 53.2 ± 7.9, 1.0 ± 0.08, 0.1 ± 0.04, and 12.6 ± 0.6, respectively (Table 2 and Figure 3e). On the other hand, the K_m (mole percent) values for wild type, G162A, G167A, and S169A were 6.6 ± 1.7, 5.8 ± 1.0, 5.9 ± 3.2, and 5.2 ± 0.1, respectively (Table 2 and Figure 3e), suggesting that substrate binding was not greatly altered in these mutants. These results provide evidence for the essential function of this P-loop-like domain of *ISC1*, and especially for the totally conserved D163 and K168, in catalysis of Isc1p.

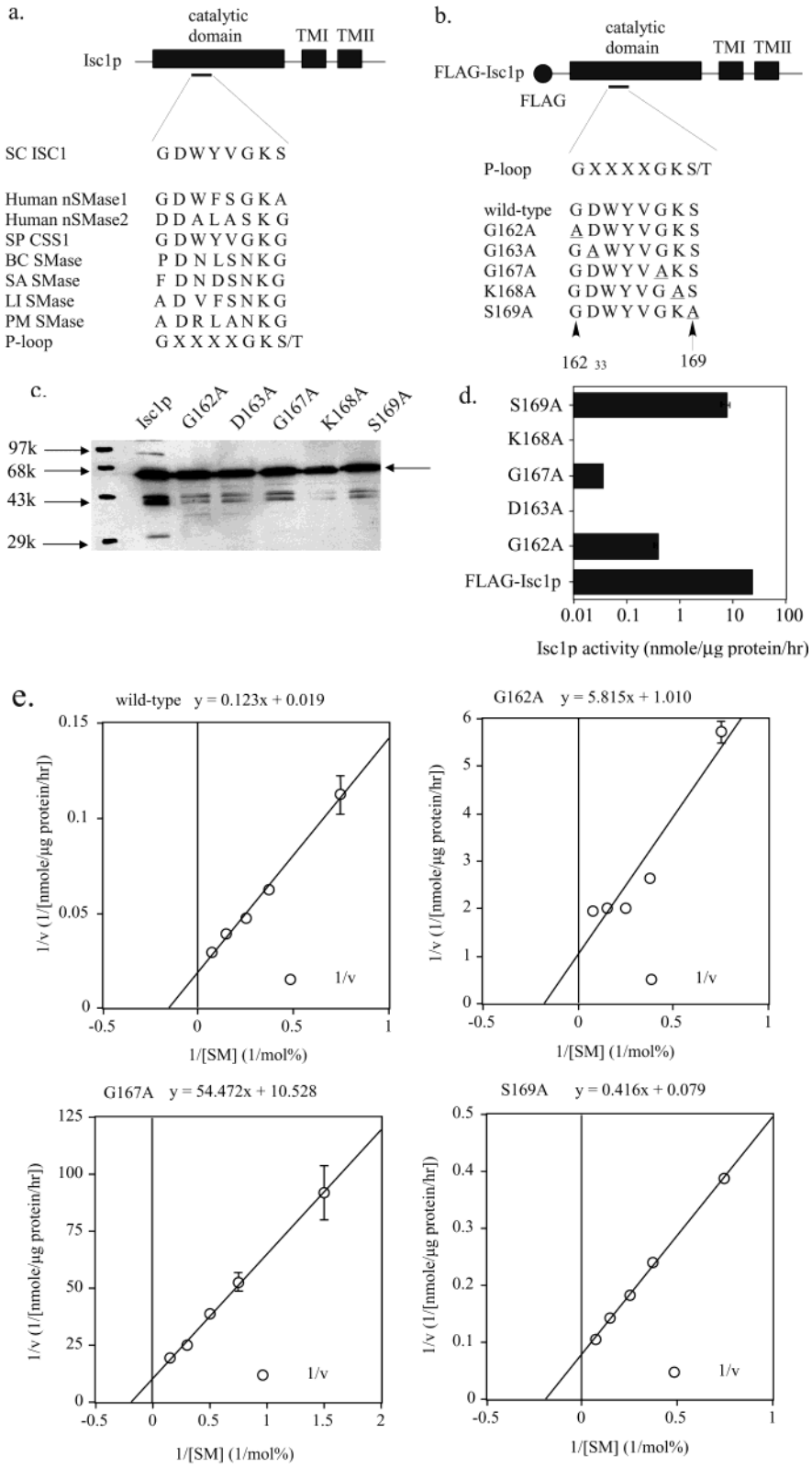

FIGURE 3: Effects of mutations in the P-loop-like domain of Isc1p on Isc1p activity. (a) Alignment of amino acid sequences of the indicated proteins with the sequence of the P-loop-like domain. (b) P-loop-like domain mutant constructs of Isc1p. Amino acid sequences are given for residues 162−169 of *ISC1*. The residues mutated from the wild type are underlined. (c) Immunoblot analysis of P-loop-like domain mutants of Isc1p with anti-FLAG antibody. Shown are two different clones of each overexpressed cell. (d) Effects of mutations in G162, D163, G167, K168, and S169 of Isc1p on Isc1p activity. Isc1p activity was measured using SM as substrate as described in Experimental Procedures. (e) Data on Isc1p activity of wild type and mutants fitting Michaelis−Menten models were analyzed using Sigma Plot to obtain the Lineweaver−Burk double reciprocal plots and their kinetic parameters. Isc1p activity on SM was measured at increasing concentrations of SM. The results are averages of triplicate experiments.

Role of the P-Loop-like Domain of ISC1 in Activation of Isc1p by Mg^{2+} and PS. We previously demonstrated that the activity of Isc1p is dependent on Mg^{2+} and phosphatidylserine (PS) (*3*). Since the sphingomyelinase activity of

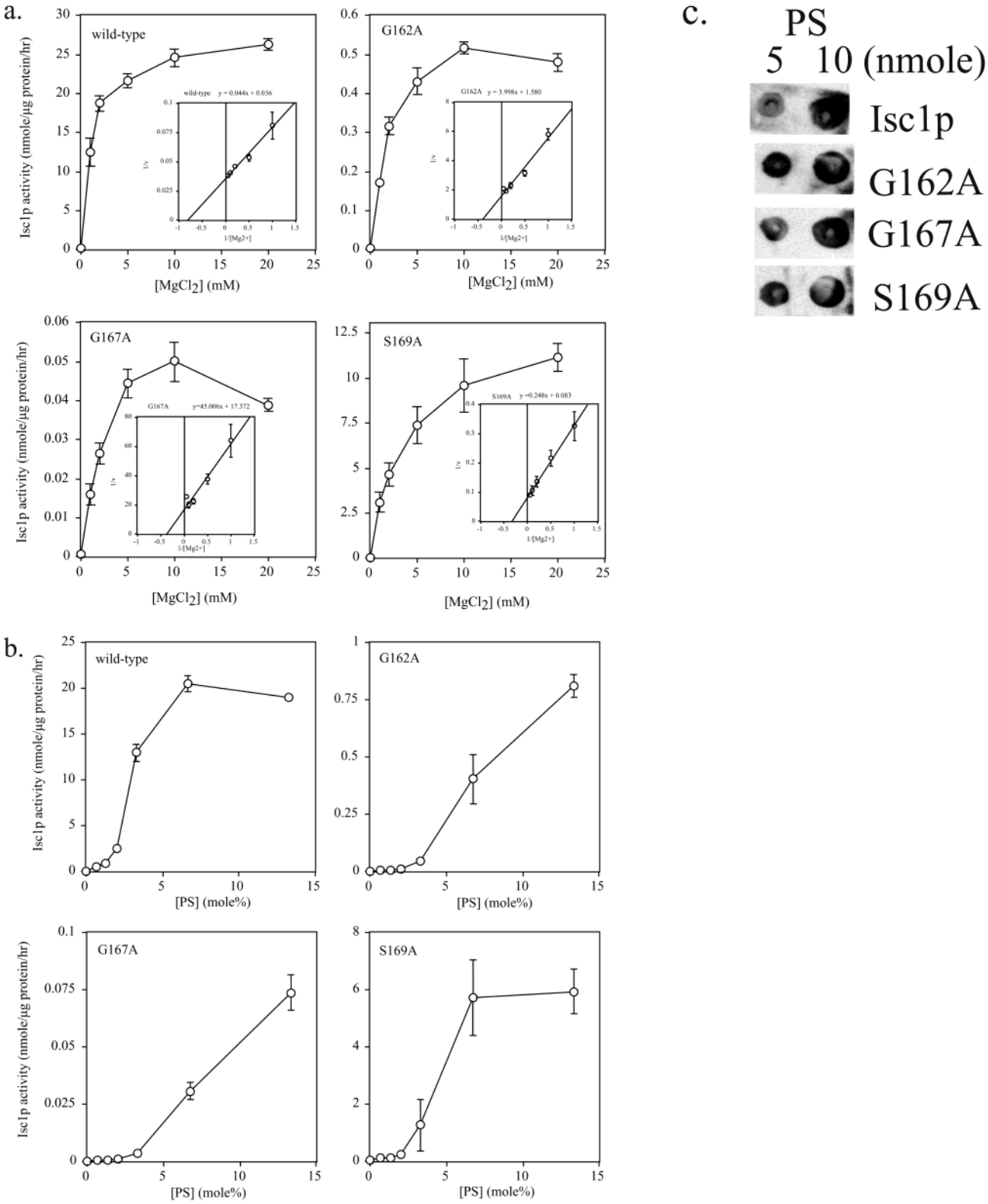

FIGURE 4: Effects of mutations in the P-loop-like domain of Isc1p on Isc1p activation by PS and Mg^{2+} and on PS binding. (a) Activity of P-loop-like domain mutants of Isc1p at various concentrations of Mg^{2+}. Isc1p activity was measured using SM as substrate as described in Experimental Procedures. The results are averages of duplicate experiments. Similar results were obtained in two different experiments. The inset shows a Lineweaver−Burk double reciprocal plot of these data, obtained using Sigma Plot data analysis under Michaelis−Menten conditions. (b) Activity of P-loop-like domain mutants of Isc1p at various concentrations of PS. Isc1p activity was measured using SM as substrate as described in Experimental Procedures. The results are averages of triplicate experiments. (c) Lipid−protein overlay assay showing the P-loop-like domain mutants binding to PS. Lipid−protein binding was identified by immunostaining with an anti-FLAG monoclonal antibody. The results are representative experiments of at least three independent experiments.

G162A, G167A, and S169A decreased compared to wild type (Table 2 and Figure 3d), we investigated the effects of Mg^{2+} and PS on activation of G162A, G167A, and S169A. Figure 4a shows the effects of Mg^{2+} on Isc1p activity of these mutants and that Isc1p activity of these mutants is also dependent on Mg^{2+}. Mg^{2+} binding constants (K_a's, milli-

Table 2: K_m and V_{max} Values of the Sphingomyelinase Activity of Isc1p in Lysates of Wild-Type, G162A, G167A, and S169A Overexpressing Cells

	wild type	G162A	G167A	S169A
K_m (mol %)	6.6 ± 1.7	5.8 ± 1.0	5.9 ± 3.2	5.2 ± 0.1
V_{max} (nmol μg^{-1} h^{-1})	53.2 ± 7.9	1.00 ± 0.08	0.10 ± 0.04	12.6 ± 0.6

molar) obtained from double reciprocal plots for wild type, G162A, G167A, and S169A were 1.04 ± 0.32 (mean ± SD), 2.35 ± 0.17, 2.73 ± 1.33, and 3.05 ± 1.00 (Figure 4a), respectively, suggesting that this P-loop-like domain may be involved in Mg^{2+} binding.

Next, we examined the effects of PS on Isc1p activity of these mutants. Recently, we demonstrated that the stimulation of the activity by PS was cooperative and that the second transmembrane domain and the C-terminus of Isc1p are required for PS binding (*3*, *9*). As shown in Figure 4b, Isc1p activity of these mutants was also dependent on PS, and the stimulation of the activity was S-shaped. Activation of wild type and S169A by PS was similar, with a maximal activity at 6.7 mol % PS and with a concentration giving 50% of the maximal response between 3 and 4 mol % PS (Figure 4b). On the other hand, activation of G162A and G167A by PS was blunted with a shift of the concentration of PS giving maximal activity to greater than 10 mol % (the mixed micelles did not sustain concentrations of PS greater than 15 mol % in the presence of sphingomyelin).

To test the effect of G162A, G167A, and S169A on the binding of Isc1p to PS, lipid−protein binding studies were carried out using the lipid−protein overlay method as described previously (*9*). Figure 4c demonstrates the binding of G162A, G167A, and S169A to PS using the lipid−protein overlay assay. As a control for nonspecific binding of protein to lipids, FLAG-tagged GFPuv was used, and the binding of FLAG-GFPuv to PS was hardly detectable (data not shown) (*9*). The binding of cell lysates from yeast cells overexpressing FLAG-*ISC1* to PS was very prominent, and cell lysates from yeast cells overexpressing G162A, G167A, and S169A bound similarly to PS, suggesting that G162A, G167A, and S169A retained the ability to interact with PS.

DISCUSSION

The results from this study define specific amino acid residue(s) and a specific domain in Isc1p required for catalysis. Individual mutagenesis of three highly conserved amino acids, E100, D234, and H334, in the catalytic domain completely abrogated Isc1p activity (Figure 2). On the basis of the comparison between bacterial sphingomyelinase and DNase I, E100 is likely to function in metal ion binding, D234 in an interaction with the phosphate group of sphingomyelin, and H344 as a general base in the catalytic reaction in *ISC1* (*7*). Recent studies have also shown through site-directed mutagenesis that these conserved amino acids in nSMase1 are also involved in catalysis (*16*).

Further site-directed mutagenesis studies showed that specific mutations of the P-loop-like domain in *ISC1* resulted in a great reduction of Isc1p activity (Figure 3d and Table 2). The V_{max} values for G162A, G167A, and S169A were reduced compared with wild type, but the K_m values for G162A, G167A, and S169A were similar to that of wild type. Recently, we reported that truncation of the P-loop-like domain completely abrogated Isc1p activity (*9*). Taken together with these results, it is suggested that the P-loop-like domain may be important for catalytic efficiency. Furthermore, the Mg^{2+} K_a values for G162A, G167, and S169A were modestly higher than that for wild type, suggesting that this P-loop-like domain may be involved in Mg^{2+} binding. Since Mg^{2+} may function by interaction with the phosphate group of the substrate, the P-loop-like domain may function to interact with substrate through a Mg^{2+}/phosphate bridge, analogous to the known structural role of P-loops in interaction with Mg^{2+}/nucleotides (*8*).

Replacement of D163 and K168 with alanine in the P-loop-like domain within the catalytic region completely abrogated Isc1p activity (Figure 3d), suggesting an essential role in catalysis for both amino acids. D163 and K168 are conserved among all sphingomyelinases from bacteria to mammals (Figure 3a), further supporting their critical role. Previously, it was reported that in the case of bacterial sphingomyelinase, D126, which corresponds to D163 in this P-loop-like domain, might be involved in substrate recognition rather than catalytic action (*15*). Another recent study suggested that D126 is located close to the active site and is involved in catalytic activity and substrate binding of bacterial sphingomyelinase (*17*). Our results also suggest that D163 is required for catalysis by Isc1p (Figure 3d). However, it could not be determined from the experiments described above whether D163 or K168 plays an important role in the catalytic activity or substrate binding in Isc1p because D163A or K168A abrogated Isc1p activity completely (Figure 3d) and K_m analysis could not be conducted. Also, direct binding studies of Isc1p to substrate have not been successful, most likely because Isc1p needs to bind PS (or anionic lipids) prior to accessing substrates; thus, whereas binding to PS can be studied, binding to substrate cannot.

As described above, the current results show that the V_{max} values for G162A, G167A, and S169A are lower compared with wild-type V_{max} (Table 2), suggesting that G162, G167, or S169 is required for catalytic efficiency of Isc1p. Recently, it has been reported that the fission yeast *S. pombe* has an *ISC1* homologue termed *CSS1*, which encodes a phospholipase C-like activity that releases ceramide from inositol phosphoceramide and mannose inositol phosphoceramide (*4*), and that one of the *css1* temperature-sensitive mutant alleles was found to contain a single point mutation of G118, which was altered to glutamic acid (*4*). This mutation of *CSS1* corresponds to G162 of *ISC1*, supporting the conclusion that this conserved residue is necessary for activity of Isc1p.

Another interesting feature of this study is that G162A and G167A required higher concentrations of PS (>13.3 mol %) to achieve maximum activation compared to wild type (Figure 3b). As shown in Figure 3c, cell lysates from yeast cells overexpressing G162A and G167A similarly bound PS when compared with wild type, suggesting that G162A and G167A also can interact with PS. These results support our recent finding that truncation of the P-loop-like domain does not affect binding to PS (*9*). Taken together with these observations, it is concluded that this P-loop-like domain is not required for PS binding. However, the reason for the requirement for higher PS of these mutants remains unknown, and further studies are needed to answer this question.

In this study, we demonstrate that amino acid residues conserved from bacterial sphingomyelinase to mammalian enzymes are essential for catalysis by Isc1p, demonstrating an evolutionary conservation of this enzyme and its mechanism. The results also identify a P-loop-like domain, which is also conserved and required for catalysis but not for interaction with activating anionic lipids such as PS.

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