

Identification of *ISC1* (*YER019w*) as Inositol Phosphosphingolipid Phospholipase C in *Saccharomyces cerevisiae**

Received for publication, August 23, 2000
Published, JBC Papers in Press, September 26, 2000, DOI 10.1074/jbc.M007721200

Hirofumi Sawai[‡]§, Yasuo Okamoto[‡]¶, Chiara Luberto[‡], Cungui Mao^{||}, Alicja Bielawska[‡], Naochika Domae[§], and Yusuf A. Hannun[‡]**

From the [‡]Department of Biochemistry and Molecular Biology and the ^{||}Ralph H. Johnson Veterans Administration Hospital and the Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425 and the [§]Department of Medicine, Osaka Dental University, 8-1 Kuzuhahanazonocho, Hirakata, Osaka 573, Japan

Sphingolipids have emerged as novel bioactive mediators in eukaryotic cells including yeast. It has been proposed that sphingomyelin (SM) hydrolysis and the concomitant generation of ceramide are involved in various stress responses in mammalian cells. The yeast *Saccharomyces cerevisiae* has inositol phosphosphingolipids (IPS) instead of SM and glycolipids, and synthesis of IPS is indispensable to its growth. Although the genes responsible for the synthesis of IPS have been identified, the gene(s) for the degradation of IPS has not been reported. Here we show that *ISC1* (*YER019w*), which has homology to bacterial neutral sphingomyelinase (SMase), encodes IPS phospholipase C (IPS-PLC). First, we observed that overexpression of *ISC1* greatly increased neutral SMase activity, and this activity was dependent on the presence of phosphatidylserine. Cells deleted in *ISC1* demonstrated negligible neutral SMase activity. Because yeast cells have IPS instead of SM, we investigated whether IPS are the physiologic substrates of this enzyme. Lysates of *ISC1*-overexpressing cells demonstrated very high PLC activities on IPS. Deletion of *ISC1* eliminated endogenous IPS-PLC activities. Labeling yeast cells with [³H]dihydrospingosine showed that IPS were increased in the deletion mutant cells. This study identifies the first enzyme involved in catabolism of complex sphingolipids in *S. cerevisiae*.

sphingomyelinase (SMase) and *de novo* synthesis. At least five SMases (acid, neutral membrane-bound, neutral cytosolic, Zn²⁺-dependent, and alkaline) exist in mammalian cells, and acid SMase and membrane-bound neutral SMase (N-SMase) have been cloned (6–10).

In the yeast *Saccharomyces cerevisiae*, many genes involved in sphingolipid metabolism have been identified, and the genes involved in the synthesis of sphingolipids such as *LCB1/LCB2* are essential for growth (11, 12). *S. cerevisiae* possesses inositol phosphosphingolipids (IPS) (inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide (M(IP)₂C)) as its complement of complex sphingolipids instead of choline phosphorylceramide (SM) and glycosphingolipids of mammals (13, 14). *AUR1*, which encodes IPC synthase, is also essential for the growth of *S. cerevisiae*, indicating that IPS may play a crucial role in yeast (15). However, the gene(s) involved in degradation of IPS in yeast have not been elucidated. Interestingly, the existence of N-SMase activity in *S. cerevisiae* was recently reported, although SM does not appear to exist in *S. cerevisiae* (16). This raised the possibility that N-SMase activity may be an *in vitro* activity of yeast IPS phospholipase C (IPS-PLC). Therefore, we investigated *YER019w*, a yeast homolog of bacterial N-SMase (7), to test this hypothesis.

Here we report that *YER019w* indeed encodes IPS-PLC. We named this gene *ISC1* for IPS-PLC. Overexpression of *ISC1* in *S. cerevisiae* dramatically increased N-SMase as well as IPC-, MIPC-, and M(IP)₂C-PLC activities. Interestingly, these activities demonstrated a strict requirement for phosphatidylserine (PS) or other acidic phospholipids in the assay. Furthermore, all of these activities were completely eliminated in the deletion mutant cells of *ISC1*. Radiolabeling experiments showed an increase of M(IP)₂C and IPC in the deletion mutant cells. Therefore, these results identify the first enzyme of *S. cerevisiae* involved in the catabolism of complex sphingolipids. The potential roles for IPS-PLC in *S. cerevisiae* are discussed.

EXPERIMENTAL PROCEDURES

Materials—*myo*-[2-³H]inositol (20 Ci/mmol) and *D-erythro*-[4,5-³H]dihydrospingosine (30 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [1-*palmitoyl*-¹⁴C]lysophosphatidylcholine (lyso-PC; 56.7 Ci/mmol), [*choline-methyl*-¹⁴C]dipalmitoylphosphatidylcholine (PC; 159 mCi/mmol), [*myo*-inositol-2-³H]phosphatidylinositol (PI; 16.5 Ci/mmol), and [9,10-³H]palmitic acid (43 Ci/mmol) were from PerkinElmer Life Sciences. Lyso-[1-*octadecyl*-³H]platelet-activating factor (lyso-PAF; 161 Ci/mmol) was from Amersham Pharmacia Biotech. [*choline-methyl*-¹⁴C]SM was synthesized as described (8). Other lipids were from Avanti Polar Lipids.

Sphingolipids such as ceramide, sphingosine, and sphingosine 1-phosphate have been proposed as intracellular mediators in various cellular responses (1–5). Ceramide has been proposed to be involved in stress responses induced by serum deprivation, heat shock, irradiation, or anticancer drugs. Two mechanisms of ceramide generation in these stresses have been reported so far: hydrolysis of sphingomyelin (SM)¹ by

* This work was supported in part by National Institutes of Health Grant GM43825 (to Y. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of the Merck Company Foundation and Banyu Fellowship Awards in Lipid Metabolism and Atherosclerosis.

** To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-4321; Fax: 843-792-4322, E-mail: hannun@musc.edu.

¹ The abbreviations used are: SM, sphingomyelin; SMase, sphingomyelinase; N-SMase, neutral sphingomyelinase; IPS, inositol phosphosphingolipid(s); IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; M(IP)₂C, mannosyldiinositol phosphorylceramide; PLC, phospholipase C; IPS-PLC, inositol phosphosphingolipid phospholipase C; PS, phosphatidylserine; lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; lyso-PAF, lyso-platelet-

activating factor; PCR, polymerase chain reaction; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl).

TABLE I

PLC activities on SM, IPC, MIPC, and M(IP)₂C in lysates of vector transfectant, *ISC1*-overexpressing cells, and wild-type cells (WT), and deletion mutant of *ISC1* (KO)

The results are means ± S.D. of triplicate experiments (nanomoles/μg/h). V, vector transfectant; Y, *ISC1*-overexpressing cells; WT, wild-type *ISC1* cells; KO, deletion mutant *ISC1* cells.

	V	Y	WT	KO
SM	0.011 ± 0.001	3.699 ± 0.185	0.013 ± 0.001	0.0007 ± 0.0001
IPC	0.006 ± 0.001	1.402 ± 0.006	0.009 ± 0.002	0
MIPC	0.011 ± 0.002	5.692 ± 0.049	0.023 ± 0.003	0
M(IP) ₂ C	0.018 ± 0.002	6.407 ± 0.296	0.034 ± 0.002	0.0011 ± 0.0011

Oligodeoxynucleotides were purchased from IDT, Inc.

Yeast Strains and Culture Media—The *S. cerevisiae* strain JK9-3dα (*MATα trp1 leu2-3 his4 ura3 ade2rme1*) (17) was used as wild-type cells. Yeast deletion mutant strains 32771 (MIPC synthase (*SUR1*) deletion mutant) and 34007 (M(IP)₂C synthase (*IPT1*) deletion mutant) were purchased from Research Genetics. Yeast extract and peptone were from Difco. Synthetic minimal medium (SD), SD/Gal, and Ura dropout supplement were purchased from CLONTECH.

Plasmids—The pYES2 yeast expression vector containing a galactose-inducible promoter was purchased from Invitrogen. cDNA of *ISC1* (*YER019w*) with the FLAG tag was generated by PCR using CGGGGTACCATTGGACTACAAGGACGACGATGATAAGATGTACAACAGAAAGACAGAGATGTTTC (primer P1, containing the sequence for the FLAG tag) and GCATTCTAGATCATTTCTCGCTCAAGAAAGTTTG as primers, and genomic DNA was extracted from JK9-3dα as template. pYES2 and the PCR product were digested by the restriction enzymes *KpnI* and *XbaI* and ligated. The sequence of the PCR product was analyzed by an ABI 377 DNA sequencer. Plasmids were transfected into yeast cells as described (17), and the expression of *ISC1* was induced by incubating the cells in synthetic complete-Ura medium containing 2% galactose overnight.

Yeast Gene Deletion—The *ISC1* gene was disrupted as described (17). Briefly, PCR was performed using GAAGCACTGAACGGGACCAACGCAATTTATGTCGACAGCTGAAGCTTCGTACGC and CTCAAGAAAGTTTGCAGGTGGTGCTCCGCTCCAGGCATAGGCCACTAGTGGATCTG (primer P2) as primers and pFA6 as template. The PCR product (100 ng) was transfected into JK9-3dα as described above, and transformed cells were selected on yeast extract/peptone/dextrose plates containing 200 μg/ml G418 (Life Technologies, Inc.). Gene disruption was confirmed by PCR using primers P1 and P2.

Preparation of IPS—Yeast lipids were extracted with solvent A (ethanol, water, diethyl ether, pyridine, and concentrated ammonia (15:15:5:1:0.018)) as described (18). Extracted lipids were base-hydrolyzed with monomethylamine reagent as described (19, 20), dried down, dissolved in chloroform/methanol/water (16:16:5), and separated by TLC with solvent B (chloroform, methanol, and 4.2 N ammonium hydroxide (9:7:2)) (21). IPC, MIPC, and M(IP)₂C were identified using [³H]inositol-labeled lipids as standards. The MIPC synthase (*SUR1*) deletion mutant (22), the M(IP)₂C synthase (*IPT1*) deletion mutant (23), and JK9-3dα were used for the preparation of IPC, MIPC, and M(IP)₂C, respectively.

Preparation of Radiolabeled IPS—For the preparation of radiolabeled IPS, yeast cells were labeled with [³H]inositol, [³H]palmitic acid, or [³H]dihydrospingosine and purified as described above.

Preparation of Lysates of Yeast Cells—Yeast cells were suspended in buffer containing 25 mM Tris (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 4 μg/ml each chymostatin, leupeptin, antipain, and pepstatin A. Cells were disrupted with glass beads as described (17). Glass beads and cell debris were removed by centrifugation at 2000 × *g* for 5 min. Protein concentration was determined using Bio-Rad protein assay reagent.

Assay of PLC Activities on SM, IPS, PC, and PI—The activity of N-SMase was examined as described (24) with modifications. Briefly, cell lysates (2–10 μl) were incubated in 100 μl of buffer containing 50 mM Tris (pH 7.5), 5 mM MgCl₂, 5 mM dithiothreitol, 0.1% Triton X-100, 10 nmol (6.7 mol %) of PS, 2.5 nmol (1.7 mol %) of unlabeled SM, and 100,000 dpm of [*choline-methyl*-¹⁴C]SM at 30 °C for 30 min. After the incubation, 0.8 ml of chloroform, 0.4 ml of methanol, and 0.2 ml of water were added according to the method of Folch *et al.* (25), and the radioactivity in a portion (400 μl) of the upper phase was measured by liquid scintillation counting. PLC activities on IPC, MIPC, M(IP)₂C, PC, and PI were examined as described for the N-SMase assay using the respective lipids (IPS labeled with [³H]inositol were used) instead of SM. For the separation of phases in assays using IPS, 0.2 ml of 1% perchloric acid was used instead of water. For M(IP)₂C, lipid extraction by the

method of Folch *et al.* (25) was repeated three times.

Lyso-PAF-PLC and Lyso-PC-PLC Assays—Lyso-PAF-PLC and lyso-PC-PLC activities were determined as described (24).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis was performed as described (26). For Western blot analysis, anti-FLAG M2 antibody (Sigma) and anti-mouse horseradish peroxidase conjugate (Santa Cruz Biotechnology) were used at 1:5000 and 1:2000 dilutions, respectively.

Purification of the Overexpressed Protein with a FLAG Tag—Lysates of *ISC1*-overexpressing cells were prepared as described above. The lysates were then incubated with 0.1% Triton X-100 for 1 h and centrifuged at 100,000 × *g* for 1 h. The supernatant was 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 0.1 M glycine (pH 3.5). The eluate was immediately neutralized with 0.025 volume of 1 M Tris (pH 8.0).

Labeling of Yeast Cells with NBD-C₆-SM—Yeast cells (2 × 10⁷) were labeled with 2.5 μM NBD-C₆-SM (Molecular Probes, Inc.) in SC medium for 1 h. Lipids were extracted by the method of Folch *et al.* (25) and separated by TLC with solvent C (chloroform, methanol, and 15 mM CaCl₂ (60:35:8)).

Radiolabeling of Yeast Cells—Yeast cells (1–2 × 10⁷) were labeled with [³H]dihydrospingosine in SC medium for the indicated times. Lipids were extracted with solvent A, base-hydrolyzed with monomethylamine reagent, and separated by TLC with solvent B. The plate was treated with ENHANCE spray (PerkinElmer Life Sciences) and exposed to x-ray film at –80 °C for 2 days. The bands for IPS were scraped and subjected to liquid scintillation counting.

RESULTS

Induction of N-SMase Activity by Overexpression of *ISC1* in *S. cerevisiae*—Recently, it was reported that the amino acid sequence of the *Isc1p* shares slight homology (~10% identity) with that of bacterial N-SMase (7). Furthermore, overexpression of mouse or human homologs of the *Isc1p* induced N-SMase activity, although lyso-PAF instead of SM is most likely the *in vivo* substrate of the human homolog (24). Whereas SM probably does not exist in yeast, a recent report showed N-SMase activity in *S. cerevisiae* (16). To elucidate whether the *Isc1p* possesses N-SMase activity, pYES2 containing the sequence of *ISC1* was constructed and transfected into JK9-3dα, and the expression of *ISC1* was induced by SC-Ura medium containing 2% galactose overnight. In initial experiments, N-SMase activity in lysates of vector transfectant or *ISC1*-overexpressing cells was negligible. Because the activity of N-SMase partially purified from rat brain was greatly stimulated by PS or other acidic phospholipids (27), PS was added to the N-SMase assay. Surprisingly, the N-SMase activities of both the vector and *ISC1*-overexpressing cells were greatly increased by PS. Furthermore, the N-SMase activity of *ISC1*-overexpressing cells was increased by >100-fold compared with that of vector transfectant in the presence of PS (Table I). In the presence of 100 μM (6.7 mol %) PS in the assay, the *K_m* and *V_{max}* for SM were 2.37 mol % and 9.2 nmol/μg/h, respectively (Table II).

Purification of the *Isc1p* Using Anti-FLAG Affinity Gel—The overexpressed protein with the FLAG tag was detected at ~55 kDa by Western blot analysis using anti-FLAG antibody (Fig. 1A). When cell lysates were fractionated by centrifugation at 100,000 × *g*, most of the overexpressed protein was detected in

TABLE II
 K_m and V_{max} values for PLC activities on SM, IPC, MIPC, and $M(IP)_2C$ in lysates of *ISC1*-overexpressing cells

	SM	IPC	MIPC	$M(IP)_2C$
K_m (mol %)	2.37	3.57	1.85	1.92
V_{max} (nmol/ μ g/h)	9.2	2.7	7.8	9.1
V_{max}/K_m	3.88	0.76	4.21	4.74

the 100,000 \times *g* pellet (Fig. 1A). Approximately 60% of N-SMase activity was recovered in the pellet, and <10% was in the supernatant (data not shown). These results demonstrate that the overexpressed protein exists in the membrane fraction. Indeed, analysis of the amino acid sequence of the Isc1p shows that it contains two putative transmembrane domains (7).

To examine whether the Isc1p itself possesses N-SMase activity, the overexpressed protein with a FLAG tag was partially purified using anti-FLAG affinity gel. One single major band at 55 kDa was detected with several minor bands on SDS-polyacrylamide gel by silver staining (Fig. 1B). This 55-kDa protein was detected with anti-FLAG antibody by Western blotting (data not shown). The specific activity of the partially purified protein on SM was 75 nmol/ μ g/h, which was >10-fold of that of the cell lysates. However, the activity of the partially purified protein was unstable. Approximately 50 and 80% of the activity was lost within 24 and 48 h at 4 °C, respectively. The activity was not preserved at -20 °C or in the presence of glycerol or albumin. Therefore, cell lysates were used in further experiments for the characterization of this enzyme.

Characterization of the Overexpressed N-SMase Activity—N-SMase activity in the lysates of *ISC1*-overexpressing cells was dependent on detergents such as Triton X-100. N-SMase activity was optimal in 0.1% Triton X-100 and was reduced in higher concentrations of Triton X-100 when fixed amounts of PS and SM were used in the assay (Fig. 2A). On the other hand, when fixed mole percents of PS and SM in Triton X-100-mixed micelles were used, N-SMase activity was almost constant between 0.1 and 1% Triton X-100 (Fig. 2B), demonstrating that the enzyme is sensitive primarily to the surface concentration of substrate and that Triton X-100 reduced the activity due to dilution of substrate and cofactor. When N-SMase activity was measured in the presence of β -octyl glucoside instead of Triton X-100, N-SMase activity was negligible without PS and was detectable only in the presence of 0.6% β -octyl glucoside with PS (data not shown). N-SMase activity was also dependent on Mg^{2+} (data not shown). Mn^{2+} stimulated N-SMase activity at 1 mM; however, higher concentrations of Mn^{2+} suppressed the activity. Ca^{2+} did not support N-SMase activity. Dithiothreitol slightly stimulated N-SMase activity (data not shown). SMase activity was maximum at pH 7.5 and greatly reduced at acidic or alkaline pH (data not shown).

Lack of N-SMase Activity in the Deletion Mutant of *ISC1*—Since N-SMase activity has been measured in *S. cerevisiae*, we investigated if the *ISC1* gene accounts for this activity. Therefore, a deletion mutant of *ISC1* (Δ *isc1*) was generated. N-SMase activity in the deletion mutant was negligible (Table I), suggesting that *ISC1* may be the only gene encoding N-SMase activity in *S. cerevisiae*.

Furthermore, we measured N-SMase activity in cells. To this end, the cells were labeled with NBD- C_6 -SM, and lipids were separated by TLC. As shown in Fig. 3, the generation of NBD- C_6 -ceramide was increased by ~3-fold in *ISC1*-overexpressing cells compared with vector transfectant. On the other hand, NBD- C_6 -ceramide was not detected in Δ *isc1* cells. These results demonstrate that the Isc1p possesses N-SMase activity in cells as well as *in vitro* and that it is the protein responsible for the

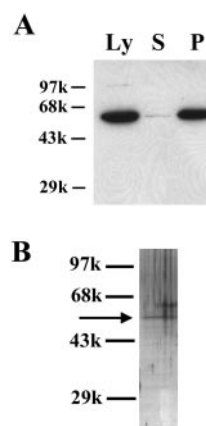


FIG. 1. **Overexpression of the Isc1p with a FLAG tag.** A, Western blot analysis with anti-FLAG antibody. Cell lysates (*Ly*), the 100,000 \times *g* supernatant (*S*), and the 100,000 \times *g* pellet (*P*) were loaded. B, purification of the FLAG-tagged protein using anti-FLAG affinity gel. The overexpressed protein was purified as described under "Experimental Procedures." The arrow indicates the overexpressed 55-kDa protein. The results are representative of two different experiments. *k*, kilodaltons.

basal activity of N-SMase in *S. cerevisiae*.

Identification of IPS as Substrates of the Isc1p—Because SM is not detectable in *S. cerevisiae*, and the major complex sphingolipids in *S. cerevisiae* are the IPS, we suspected that IPS might be substrates for the Isc1p. To examine this, 3H -labeled IPS (IPC, MIPC, and $M(IP)_2C$) were purified from yeast cells and used as substrates instead of SM in the assay. Lysates of *ISC1*-overexpressing cells showed a greatly increased hydrolytic activity on these lipids (Table I). The *in vitro* activities on IPC, MIPC, and $M(IP)_2C$ in the overexpressing cells were increased by 230-, 510-, and 360-fold, respectively, compared with those in vector cells.

When IPC purified from cells labeled with [3H]palmitic acid was used as a substrate, the product corresponded to the standard of (phyto)ceramide on TLC (Fig. 4). Also, when $M(IP)_2C$ labeled with [3H]dihydrosphingosine was used as a substrate, the product was (phyto)ceramide (data not shown). These results demonstrate that the activity was of the PLC-type.

Next, the K_m and V_{max} for these lipids were determined (Table II). The K_m values for $M(IP)_2C$ and MIPC were approximately half of that for IPC, and the V_{max} values for $M(IP)_2C$ and MIPC were ~3.4-fold and 2.9-fold of that for IPC, respectively, suggesting that $M(IP)_2C$ and MIPC are better substrates for the Isc1p than IPC, as further shown by comparison of the V_{max}/K_m ratios. The K_m and V_{max} for $M(IP)_2C$ and MIPC were almost the same as those for SM (Table II).

Furthermore, lysates of the deletion mutant of *ISC1* showed negligible activities on IPS (Table I). These results suggest that *ISC1* may be the only gene that encodes IPS-PLC activity in *S. cerevisiae*, at least under these growth conditions.

Other phospholipids were also examined as substrates. PLC activity on PC was 0.002 ± 0.001 nmol/ μ g/h in lysates of both vector transfectant and *ISC1*-overexpressing cells, suggesting that PC is not a substrate of this enzyme. PLC activity on PI was undetectable in lysates of vector transfectant and not significantly increased in lysates of *ISC1*-overexpressing cells (0.001 ± 0.001 nmol/ μ g/h), suggesting that PI is not a substrate. Moreover, the human homolog of bacterial N-SMase demonstrated PLC activities on lyso-PAF and lyso-PC (24); therefore, lyso-PAF and lyso-PC were examined as substrates of this enzyme. PLC activity on lyso-PAF was 0.013 ± 0.005 and 0.011 ± 0.016 nmol/ μ g/h, and PLC activity on lyso-PC was 0.001 ± 0.001 and 0.003 ± 0.002 nmol/ μ g/h in lysates of vector transfectant and *ISC1*-overexpressing cells, respectively.

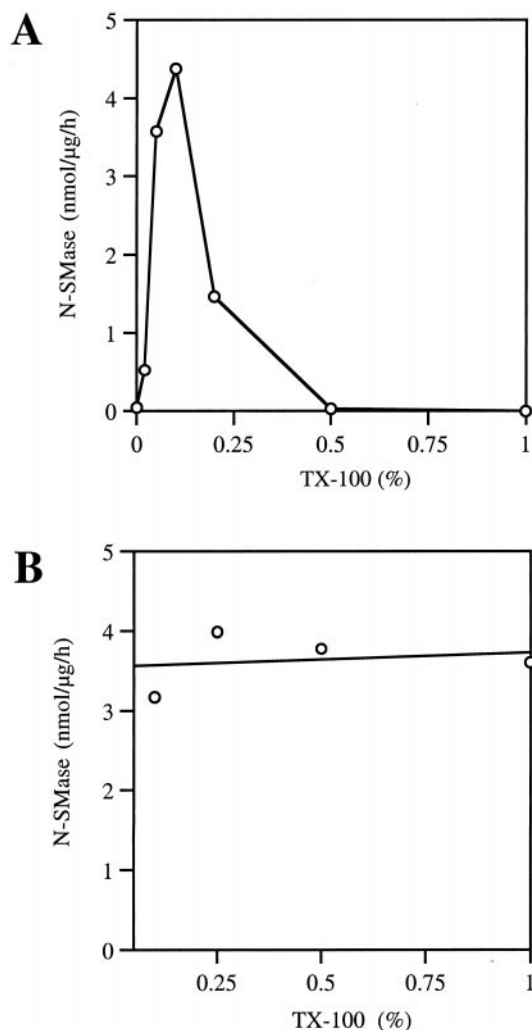


FIG. 2. **Effect of Triton X-100 on N-SMase activity.** A, N-SMase activity at various concentrations of Triton X-100 (TX-100). Fixed amounts of SM (10 nmol) and PS (10 nmol) were used in the assay. The results are means of duplicate experiments. Similar results were obtained in two different experiments. B, N-SMase activity at fixed mole percents of SM (1.7 mol %) and PS (6.7 mol %) in Triton X-100. The results are means of duplicate experiments. Similar results were obtained in two different experiments.

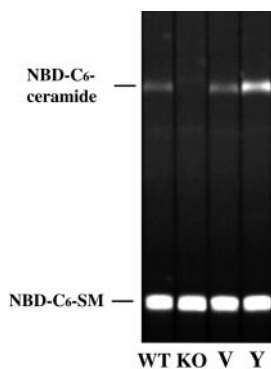


FIG. 3. **Detection of N-SMase activity in cells by labeling with NBD-C₆-SM.** Yeast cells (2×10^7) were labeled with $2.5 \mu\text{M}$ NBD-C₆-SM in SC medium for 1 h. Lipids were extracted by the method of Folch *et al.* (25) and separated by TLC as described under "Experimental Procedures." WT, wild type cells; KO, deletion mutant of *ISC1*; V, vector transfectant; Y, *ISC1*-overexpressing cells.

These results suggest that neither lyso-PAF nor lyso-PC is a substrate of this enzyme.

Characterization of the Overexpressed IPS-PLC Activi-

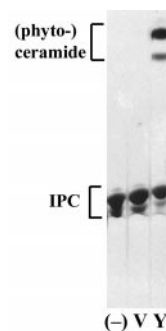


FIG. 4. **Identification of the product as (phyto)ceramide by the action of the Isc1p on IPC.** IPC purified from cells labeled with [^3H]palmitic acid was used as a substrate. After incubation without lysates (-) or with lysates of vector transfectant (V) or *ISC1*-overexpressing cells (Y), lipids were extracted by the method of Folch *et al.* (25) and were separated by TLC with solvent B.

ties—To characterize IPS-PLC activities, MIPC was used as a substrate in further experiments. PLC activity on MIPC was dependent on Mg^{2+} , and the K_m for Mg^{2+} was 2.4 mM (Fig. 5A). Mn^{2+} stimulated the activity at 1 mM; however, the activity was decreased at higher concentrations of Mn^{2+} . Ca^{2+} did not support the activity. PLC activity on MIPC was maximum at pH 7.5 and greatly reduced at acidic or alkaline pH (Fig. 5B).

Stimulation of IPS-PLC Activities by PS—As mentioned above, PLC activities on IPS as well as SM were dependent on the presence of PS in the assay. When 0.1% Triton X-100 was used in the assay, $5 \mu\text{M}$ (0.33 mol %) PS only slightly induced PLC activity on MIPC, and stimulation of the activity was maximum at $100 \mu\text{M}$ (6.7 mol %) PS (Fig. 6A). Because the stimulation of the activity by PS was S-shaped, a Hill plot was performed. As shown in Fig. 6B, the Hill coefficient was ~ 2.0 , suggesting that two or more molecules of PS interact with the Isc1p to stimulate IPS-PLC activities in a cooperative manner. When various concentrations of PS were used in the assay, a double-reciprocal plot showed that the K_m remained constant, whereas the V_{max} was increased at higher concentrations of PS (Fig. 6, C and D). Other acidic phospholipids such as PI and cardiolipin also stimulated PLC activity on MIPC, but less effectively (Fig. 6E). Phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid only slightly increased the activity (Fig. 6E).

Effect of *ISC1* Overexpression or Deletion on the Radiolabeling of IPS in *S. cerevisiae*—To examine the effect of the Isc1p on IPS metabolism in cells, we performed radiolabeling experiments with [^3H]dihydrosphingosine. When wild-type and deletion mutant cells of *ISC1* were labeled with [^3H]dihydrosphingosine, the levels of $\text{M}(\text{IP})_2\text{C}$ and IPC were increased in the deletion mutant compared with the wild type (the radioactivities were 18,000 and 27,000 dpm for $\text{M}(\text{IP})_2\text{C}$ and 28,000 and 36,000 dpm for IPC in the wild type and deletion mutant, respectively) (Fig. 7A). Interestingly, the level of MIPC was not increased in the deletion mutant compared with the wild type. These data demonstrate that the Isc1p functions as IPS-PLC in cells. On the other hand, when vector transfectant and *ISC1*-overexpressing cells were labeled with [^3H]dihydrosphingosine, the levels of IPS were not apparently decreased in *ISC1*-overexpressing cells compared with vector transfectant (Fig. 7B). Similar results were obtained by labeling the cells with [^3H]inositol (data not shown).

DISCUSSION

SM hydrolysis induced by SMases has been reported to be involved in various signal transduction and cell regulatory pathways in mammalian cells. N-SMase activity was also detected in yeast cells, although SM does not exist in yeast (16).

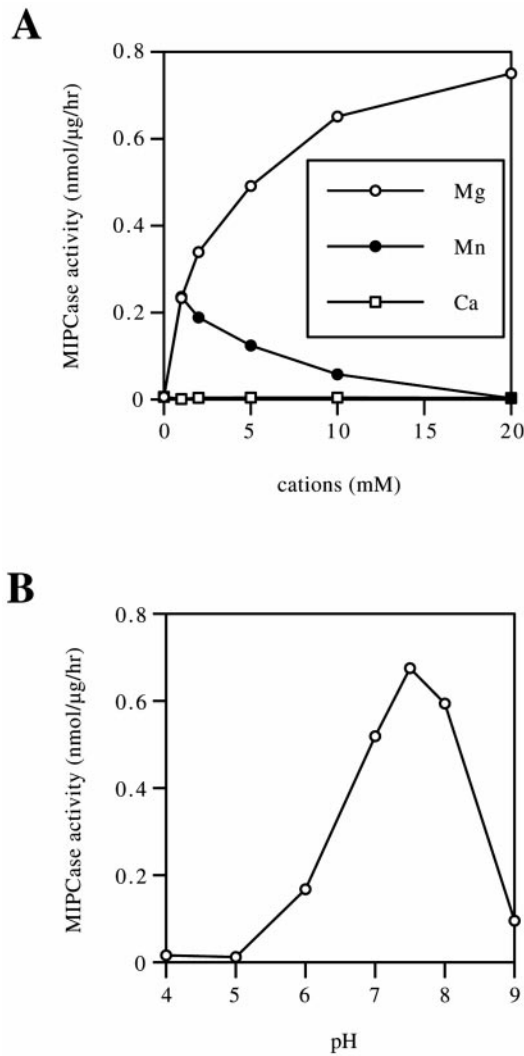


FIG. 5. Effects of cations (A) and pH (B) on IPS-PLC activity. A, the PLC activity on MIPC was measured at various concentrations of $MgCl_2$ (○), $MnCl_2$ (●), or $CaCl_2$ (□). The results are means of duplicate experiments. Similar results were obtained in two different experiments. B, the PLC activity on MIPC was measured at various pH values. The following buffers were used: pH 4–5, acetate; pH 6, phosphate; pH 7–8, Tris; and pH 9, glycine. The results are means of duplicate experiments. Similar results were obtained in two different experiments.

IPS (IPC, MIPC, and $M(IP)_2C$) are the major sphingolipids in yeast. Here we demonstrated that *ISC1* (*YER019W*) encodes IPS-PLC in *S. cerevisiae*.

It was previously shown that the *Isc1p* shares slight homology in amino acid sequence with bacterial N-SMases (7). Therefore, we suspected that it would be a good candidate for a yeast phospholipase that also acts on SM. Indeed, we detected a great increase of N-SMase activity in lysates of *ISC1*-overexpressing cells. Furthermore, this activity hydrolyzed IPC, MIPC, and $M(IP)_2C$ *in vitro*. Interestingly, the activity was completely dependent on PS (or other acidic phospholipids such as PI and cardiolipin). N-SMase activity purified from rat brain was also greatly stimulated by PS, although N-SMase activity in crude lysates of rat brain was not much increased by PS (27). On the other hand, the activity of the human homolog of bacterial N-SMases was only slightly (up to 2-fold) stimulated by PS,² although the amino acid sequence of this protein shows ~30% homology to that of the *Isc1p*.

² H. Sawai, and Y. A. Hannun, unpublished observations.

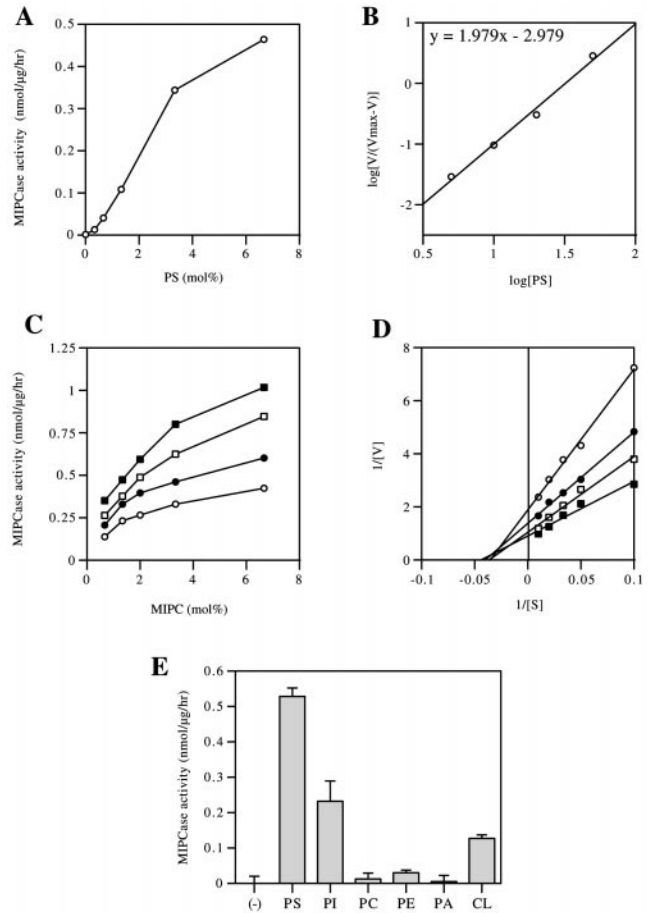


FIG. 6. Dependence of IPS-PLC activity on PS or other acidic phospholipids. A, the PLC activity on MIPC was measured at various concentrations of PS. The results are means of duplicate experiments. Similar results were obtained in two different experiments. B, shown is a Hill plot of the data in A. C and D, shown is the substrate dependence and the double-reciprocal plot of PLC activity on MIPC at various concentrations of PS: 100 μM (6.7 mol %; ■), 50 μM (3.3 mol %; □), 40 μM (2.7 mol %; ●), and 30 μM (2.0 mol %; ○). E, the PLC activity on MIPC was measured in the absence (-) or presence of various phospholipids at 100 μM (6.7 mol %). PE, phosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin. The results are means of duplicate experiments. Similar results were obtained in two different experiments.

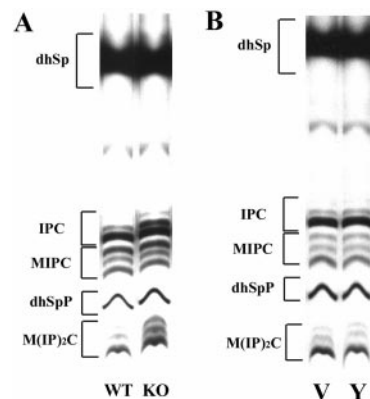


FIG. 7. Radiolabeling experiments with [³H]dihydrosphingosine. A, wild-type cells (WT) and deletion mutant of *ISC1* (KO) were labeled with 1 μCi of [³H]dihydrosphingosine for 30 min. The results are representative of three different experiments. B, vector transfectant (V) and *ISC1*-overexpressing cells (Y) were labeled with 1 μCi of [³H]dihydrosphingosine for 30 min. TLC was performed as described under "Experimental Procedures." The results are representative of two different experiments. *dhSp*, dihydrosphingosine; *dhSpP*, dihydrosphingosine 1-phosphate.

The Hill coefficient for PS of the Isc1p was ~2.0, suggesting that two or more molecules of PS interact cooperatively with the Isc1p to stimulate its activity. The precise mechanism by which PS stimulates IPS-PLC activity awaits the elucidation of PS-binding sites in the protein. It also remains to be elucidated whether PS (or other acidic phospholipids) functions as an activating factor of IPS-PLC in cells. Colocalization of the protein and PS (or other phospholipids) may be critical for the activation of IPS-PLC, and IPS-PLC activity may be induced by the increase in lipid cofactor upon cell stimulation.

The deletion mutant of *ISC1* almost completely lost PLC activities for all IPS (IPC, MIPC, and M(IP)₂C), indicating that *ISC1* is the only gene encoding IPS-PLC activities in *S. cerevisiae*. This result is in contrast with the fact that distinct genes encode the enzymatic activities for the synthesis of IPC, MIPC, and M(IP)₂C and is distinct from the fact that mammalian cells have more than one SMase. The PLC activity of the Isc1p was specific to IPS and SM because PLC activity on PC, lyso-PC, or PI was much lower than that on IPS in lysates of *ISC1*-overexpressing cells. Lyso-PAF was not a substrate for the Isc1p, although the human homolog of the Isc1p showed high activity on lyso-PAF both *in vitro* and in cells (24). These results suggest that the mammalian homolog of the Isc1p acquired another function (*i.e.* lyso-PAF-PLC activity) during the evolutionary process from yeast to mammals. It is also possible that another mammalian homolog of the Isc1p exists with specific activity for SM depending on PS as a cofactor.

Although cell labeling experiments showed increased levels of M(IP)₂C and IPC, the level of MIPC was not increased in the deletion mutant of *ISC1* compared with wild-type cells, and the levels of IPS were not apparently decreased in *ISC1*-overexpressing cells compared with vector transfectant. These results would be explained by different possibilities. First, this would be explained by counter-regulatory metabolic changes such that enzymes involved in *de novo* synthesis of IPS may be influenced by the deletion or overexpression of *ISC1* to compensate for the change in IPS-PLC activity. Second, the overexpressed Isc1p may not exert full activity due to the limitation of key activator(s) in cells. In accordance with this notion, the results obtained by labeling the cells with NBD-C₆-SM showed only a 3-fold increase in NBD-C₆-ceramide, whereas N-SMase activity was increased >100-fold *in vitro* in *ISC1*-overexpressing cells compared with vector transfectant. Finally, the overexpressed Isc1p may not have access to most of its substrates due to distinct localization because most IPS exist in the plasma membrane (28), whereas it is highly possible that the Isc1p exists in the endoplasmic reticulum judging from the fact that the Isc1p shares high homology in the putative transmem-

brane domains with its mouse homolog, which was recently shown to be localized in the endoplasmic reticulum (29). This may explain why the levels of IPS were not decreased in *ISC1*-overexpressing cells compared with vector transfectant. However, the localization of the Isc1p remains to be elucidated. In conclusion, this study identifies the yeast IPS-PLC as the first and possibly only enzyme involved in the catabolism of yeast complex sphingolipids.

Acknowledgment—We thank Dr. Samer El Bawab for helpful discussions.

REFERENCES

- Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 3125–3128
- Hannun, Y. A. (1996) *Science* **274**, 1855–1859
- Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., Kolesnick, R. N. (1996) *Nature* **380**, 75–79
- Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature* **381**, 800–803
- Perry, D. K., and Hannun, Y. A. (1998) *Biochim. Biophys. Acta* **1436**, 233–243
- Quintern, L. E., Schuchman, E. H., Levrano, O., Suchi, M., Ferlinz, K., Reinke, H., Sandhoff, K., and Desnick, R. J. (1989) *EMBO J.* **8**, 2469–2473
- Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M., and Stoffel, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3638–3643
- Okazaki, T., Bielawska, A., Domae, N., Bell, R. M., and Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 4070–4077
- Schissel, S. L., Schuchman, E. H., Williams, K. J., and Tabas, I. (1996) *J. Biol. Chem.* **271**, 18431–18436
- Duan, R. D., Nyberg, L., and Nilsson, A. (1995) *Biochim. Biophys. Acta* **1259**, 49–55
- Dickson, R. C., and Lester, R. L. (1999) *Biochim. Biophys. Acta* **1426**, 347–357
- Dickson, R. C., and Lester, R. L. (1999) *Biochim. Biophys. Acta* **1438**, 305–321
- Steiner, S., Smith, S., Waechter, C. J., and Lester, R. L. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **64**, 1042–1048
- Smith, S. W., and Lester, R. L. (1974) *J. Biol. Chem.* **249**, 3395–3405
- Nagiec, M. M., Nagiec, E. E., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1997) *J. Biol. Chem.* **272**, 9809–9817
- Ella, K. M., Qi, C., Dolan, J. W., Thompson, R. P., and Meier, K. E. (1997) *Arch. Biochem. Biophys.* **340**, 101–110
- Mao, C., Wadleigh, M., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1997) *J. Biol. Chem.* **272**, 28690–28694
- Hanson, B. A., and Lester, R. L. (1980) *J. Lipid Res.* **21**, 309–315
- Clarke, N. G., and Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306
- Mao, C., Saba, J. D., and Obeid, L. M. (1999) *Biochem. J.* **342**, 667–675
- Wells, G. B., Dickson, R. C., and Lester, R. L. (1998) *J. Biol. Chem.* **273**, 7235–7243
- Beeler, T. J., Fu, D., Rivera, J., Monaghan, E., Gable, K., and Dunn, T. M. (1997) *Mol. Gen. Genet.* **255**, 570–579
- Dickson, R. C., Nagiec, E. E., Wells, G. B., Nagiec, M. M., and Lester, R. L. (1997) *J. Biol. Chem.* **272**, 29620–29625
- Sawai, H., Domae, N., Nagan, N., and Hannun, Y. A. (1999) *J. Biol. Chem.* **274**, 38131–38139
- Folch, J., Lees, M., and Stanley, G. H. S. (1957) *J. Biol. Chem.* **226**, 497–509
- Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H., and Domae, N. (1997) *J. Biol. Chem.* **272**, 2452–2458
- Liu, B., Hassler, D. F., Smith, G. K., Weaver, K., and Hannun, Y. A. (1998) *J. Biol. Chem.* **273**, 34472–34479
- Patton, J. L., and Lester, R. L. (1991) *J. Bacteriol.* **173**, 3101–3108
- Fensome, A. C., Rodrigues-Lima, F., Josephs, M., Paterson, H. F., and Katan, M. (2000) *J. Biol. Chem.* **275**, 1128–1136