Effect of Membrane Perturbants on the Activity and Phase Distribution of Inositol Phosphorylceramide Synthase; Development of a Novel Assay

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ABSTRACT: The effect of 26 different membrane-perturbing agents on the activity and phase distribution of inositol phosphorylceramide synthase (IPC synthase) activity in crude *Candida albicans* membranes was investigated. The nonionic detergents Triton X-100, Nonidet P-40, Brij, Tween, and octylglucoside all inactivated the enzyme. However, at moderate concentrations, the activity of the Triton X-100- and octylglucoside-solubilized material could be partially restored by inclusion of 5 mM phosphatidylinositol (PI) in the solubilization buffer. The apparent molecular mass of IPC synthase activity solubilized in 2% Triton X-100 was between 1.5×10^6 and 20×10^6 Da, while under identical conditions, octylglucoside-solubilized activity remained associated with large presumably membrane-like structures. Increased detergent concentrations produced more drastic losses of enzymatic activity. The zwitterionic detergents Empigen BB, *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB), Zwittergent 3-10, and amidosulfobetaine (ASB)-16 all appeared capable of solubilizing IPC synthase. However, these agents also inactivated the enzyme essentially irreversibly. Solubilization with lysophospholipids again resulted in drastic losses of enzymatic activity that were not restored by the inclusion of PI. Lysophosphatidylinositol also appeared to compete, to some extent, with the donor substrate phosphatidylinositol. The sterol-containing agent digitonin completely inactivated IPC synthase. By contrast, sterol-based detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), and taurodeoxycholate (tDOC) had little or no effect on the enzyme activity. The IPC synthase activity in *C. albicans* membranes remained largely intact and sedimentable at CHAPS concentrations (4%) where >90% of the phospholipids and 60% of the total proteins were extracted from the membranes. At 2.5% CHAPS, a concentration where approximately 50% of the protein and 80% of the phospholipids are solubilized, there was no detectable loss of enzyme activity, and it was found that the detergent-treated membranes had significantly improved properties compared to crude, untreated membranes as the source of IPC synthase activity. In contrast to assays utilizing intact membranes or Triton X-100 extracts, assays using CHAPS- or tDOC-washed membranes were found to be reproducible, completely dependent on added acceptor substrate (C_6−7-nitro-2-1,3-benzoxadiazol-4-yl (NBD)−ceramide), and >95% dependent on added donor substrate (PI). Product formation was linear with respect to both enzyme concentration and time, and transfer efficiency was improved more than 20-fold as compared to assays using crude membranes. Determination of kinetic parameters for the two IPC synthase substrates using CHAPS-washed membranes resulted in K_m values of 3.3 and 138.0 μM for C_6−NBD−ceramide and PI, respectively. In addition, the donor substrate, PI, was found to be inhibitory at high concentrations with an apparent K_i of 588.2 μM.

Sphingolipids are an important component of the fungal plasma membrane (*1*, *2*). Although sphingolipids comprise only inositol phosphorylceramide (IPC)[1] and its mannosylated derivatives in the yeast *Saccharomyces cerevisiae*, other fungi typically also contain various species of glycosphingolipids (*3*, *4*). Together sphingolipids may constitute 16% of the total lipids in the fungal plasma membrane (*5*, *6*). Several other fungal species, including *Candida*, *Cryptococcus*, and *Histoplasma* species, also appear to contain primarily IPC-based sphingolipids (*3*, *7*). Inositol phospho-

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[1] Abbreviations: ASB, amidosulfobetaine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DDMAB, *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate; GPI, glycosylphosphatidylinositol; IPC, inositolphosphoryl ceramide; NBD, (7-nitro-2-1,3-benzoxadiazol-4-yl); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SAR, structure−activity relationship; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; tDOC, taurodeoxycholate; TMD, transmembrane domain.

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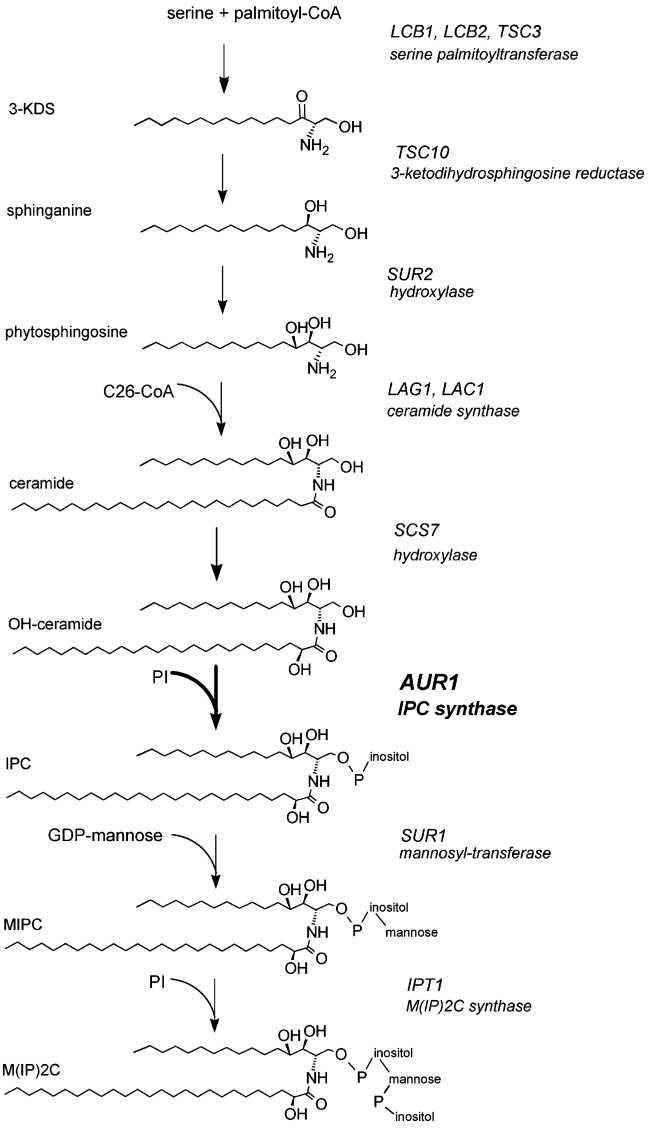

FIGURE 1: The sphingolipid biosynthesis pathway in *S. cerevisiae*. Genes corresponding to identified enzyme activities are indicated in italics.

rylceramide synthase (IPC synthase) catalyzes the transfer of inositolphosphate from phosphatidylinositol to phytoceramide in the fungal sphingolipid biosynthesis pathway (see Figure 1). The product generated by this reaction, inositol phosphorylceramide (IPC) may subsequently be further modified by the conjugation of mannose and an additional inositolphosphate moiety (reviewed in ref *2*). Although there are significant similarities between fungal and mammalian cells through the phytoceramide synthesis step in this pathway, the addition of inositolphosphate is a decidedly fungi-specific reaction. Mammalian cells instead conjugate phosphocholine to the 1-hydroxyl group on ceramide to form sphingomyelin (*8*).

The uniqueness (for fungi) of the IPC synthase-catalyzed reaction, coupled with the fact that mutational studies have demonstrated that the enzyme is essential in fungi, makes IPC synthase an attractive target for antifungal drugs (*9*). Further supporting this notion is the recent identification of several potent antifungal natural product compounds that all target IPC synthase (e.g., refs *9*−*13*).

IPC synthase is an integral membrane protein believed to be localized in the Golgi complex (*14*). A *S. cerevisiae* gene, *AUR1*, encoding the enzyme has been cloned. Sequence analysis of this gene yielded a predicted molecular mass of 45 193 Da and suggests that the molecule contains six or seven transmembrane domains (TMDs) (*9*). Western blotting experiments indicate a molecular mass of 45 kDa, suggesting that the protein does not contain extensive posttranslational modifications. A putative active site domain believed to be facing the Golgi lumen has been identified just upstream of the most COOH terminal TMD. Mutation of histidine 294 in this domain results in nonviable haploid cells (*14*).

Published assays for IPC synthase activity have significant limitations. These are, to a large extent, due to the use of crude membrane preparations or Triton X-100 extracts as the enzyme source (e.g., refs *10*, *11*, *15*, and *16*). Since the yeast Golgi membrane contains considerable amounts of phosphatidylinositol (*17*), the use of crude membranes results in an assay in which the donor substrate concentration is difficult to control. Moreover, the assay product, IPC, which presumably is incorporated into the (same) enzyme-containing crude membrane, appears to accumulate in that compartment and cause a product inhibition or back-up that gradually reduces reaction velocity and eventually blocks product formation completely (see below). Although IPC synthase can be solubilized in Triton X-100, this is accompanied by a significant (typically more that 80%) loss in activity. The activity loss can be reduced significantly by inclusion of excess PI in the solubilization buffer, but this again results in an assay where the concentration of the donor substrate is difficult to control (*11*, *15*, *16*). Consequently, neither crude-membrane- nor current detergent-extract-based assays are suitable for kinetic studies of IPC synthase substrates or for evaluation of (putative) inhibitors.

This report describes the effect(s) of a number membrane perturbants on the activity and phase distribution of IPC synthase, as well as a novel IPC synthase assay that, to a large extent, circumvents the problems discussed above. The assay, which is based on the use of detergent-treated membranes, is reproducible, more efficacious than previous assays, dependent on the addition of both substrates, and linear until exhaustion of substrate. Kinetic parameters for the substrates C_6−NBD−ceramide and phosphatidylinositol are presented.

MATERIALS AND METHODS

Materials. Brij 35, Brij 58, CHAPS, CHAPSO, digitonin, dodecylmaltoside, Empigen BB, Nonidet P-40, octyl β-D-glucopyranoside, lysophsophatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol, octyl β-D-thioglucopyranoside, oleic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, SDS, taurodeoxycholate, Triton X-100, Triton N-60, Tween 20, Tween 80, and Zwittergent 3-10 were from Sigma. Pefabloc was from Roche Molecular Biochemicals. Optiprep was purchased from Nycomed Pharma AS. Tris−glycine SDS−PAGE gels were from Invitrogen. Sepharose 4B, Sephacryl S200, Sephacryl S300, Blue Dextran, and ^{3}H-mannose were purchased from Amersham Biosciences. Silica gel plates were from Analtech. AG4-X4 resin was from BioRad. C_6−NBD−ceramide was from Cayman Chemical. Purified phosphatidylcholine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and lysophosphatidylglycerol were

from Avanti Polar Lipids. Cyclohexyl-*n*-hexyl-*β*-D-maltoside, *n*-decyl-*β*-D-thiomaltoside, *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB) and amidosulfobetaine (ASB)-16 were from Merck/Calbiochem.

Preparation of Crude Membranes. Crude *Candida albicans* (ATCC 38247) membranes were prepared essentially as described by Hendershot et al. (*18*). The protein concentration of the washed membrane suspension was determined according to Lowry et al. (*19*) and adjusted to 10 mg/mL. The membranes were stored in 50 mM Tris-HC1, pH 7.5, 5 mM $MgC1_2$, 20% glycerol, and 1 mM Pefabloc at −80 °C until use.

Solubilization of Membranes and IPC Synthase Activity in Detergents. Solubilization of crude membranes in detergents and lysophospholipids was done by suspending sedimented membrane pellets in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20% (v/v) glycerol, and 1 mM Pefabloc, containing 1−2.5% detergent or lysophospholipid at protein concentrations of 3−10 mg/mL for a final detergent-to-protein ratio of 1:1 to 8:1 (mass/mass). All detergent concentrations are given as percent (w/v). Following incubation for 60 min on ice, nonsolubilized material was sedimented at 150 000 × *g* for 60 min; the soluble supernatant fraction was removed and the sedimented; insoluble material was suspended in the original volume of buffer without detergent. Total enzyme activity was determined in the supernatant and pellet fractions as described below.

Solubilization of crude membranes in Triton X-100, 0.3−4% CHAPS, and 2.5−5% taurodeoxycholate (tDOC) was done as described above but using the indicated (see applicable experiments) concentrations of detergent and protein. Following incubation for 60 min on ice, separation of soluble and insoluble material and assays for IPC synthase activity were carried out as described above.

Reconstitution of IPC synthase activity in detergent-solubilized fractions was accomplished by incubating aliquots in the presence of 1−5 mM PI on ice for approximately 3 h.

The effect of phospholipids on the IPC synthase activity in intact membranes was investigated by incubating 5 μL of intact membranes (10 mg/mL total protein) in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM EDTA with 2 μL of 12.5 mM phospholipid for 20 min prior to assay for IPC synthase activity. Phosphatidylethanolamine (PE), which is insoluble in water, was solubilized in 5% Triton X-100, producing a final Triton X-100 concentration of 0.25% in the assay.

Density Gradient Centrifugation. A total of 250 μL of *C. albicans* cell extract (equivalent to 45 OD_{600} units of cells) containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 was incubated 30 min on ice and combined with 500 μL of OptiPrep (40% v/v OptiPrep, final). A total of 1.2 mL of 30% (v/v) OptiPrep in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 and 0.2 mL of the same buffer were sequentially layered onto the enzyme-containing, 40% (v/v) solution using a 1 mL syringe. The sample was centrifuged for 2 h at 200 000 × *g* in a TLS 55 rotor at 4 °C, and 400 μL fractions were collected from the top of the gradient. IPC synthase activity was reconstituted by the addition of 1 mM PI and incubation at 4 °C overnight. Ten microliter aliquots were assayed for activity.

Electrophoresis. Aliquots from the OptiPrep gradient fractions were precipitated as outlined by Wessel and Flügge (*20*) and separated on 10% (w/v) Tris−glycine SDS−PAGE gels (125 V; 100 min). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (25 V; 90 min) and blotted with a polyclonal antibody raised against a peptide in the sequence of the *C. albicans* GPI-anchored cell surface protein PHR1 (K. Peterson and Å. Elhammer, unpublished data). The blot was visualized using the Western Breeze chromagenic Western blotting immunodetection system (Invitrogen).

Column Chromatography. A 500 μL aliquot of membrane suspension (2 mg/mL total protein) in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 1 mM PI was incubated with 1% Triton X-100 or octylglucoside for 30 min at 4 °C and applied to a 0.7 cm × 16 cm Sepharose 4B column equilibrated with 50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$, containing 0.1% detergent. Ten 0.5 mL fractions were collected and assayed for IPC synthase activity. Included and excluded volumes were determined using ^{3}H-labeled mannose and Blue Dextran 2000, respectively.

Two milliliter aliquots of membrane suspension (10 mg/mL total protein) in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, and 5 mM EDTA containing 2% Triton X-100 and 5 mM PI were incubated for 30 min at 4 °C. The samples were applied to a 1.6 cm × 100 cm Sephacryl S200 or S300 column equilibrated with the above buffer containing 0.2% CHAPS and 0.25 mM PI. The columns were eluted at 0.15−0.3 mL/min; 2.5 mL fractions were collected, and 10 μL of each fraction was assayed for IPC synthase activity. Included and excluded volumes were determined using ^{3}H-labeled mannose and Blue Dextran 2000, respectively.

Preparation of Detergent-Washed Membranes. Crude membranes (10 mg/mL) were washed in 2.5% CHAPS for 60−90 min at 4 °C. The membranes were sedimented at 100 000 × *g* for 60 min at 4 °C. The supernatant was removed, and the detergent-washed membranes were suspended in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20% (v/v) glycerol, and 1 mM Pefabloc. The protein concentration was determined and adjusted to 10 mg/mL, and the detergent-washed membranes were stored at −80 °C. For preparation of two- and three-times washed membranes, the protein concentration was readjusted to 10 mg/mL and detergent concentration to 2.5% between each wash step.

Extraction of Lipids. Total lipids were extracted from crude and detergent-washed membranes as described by Bligh and Dyer (*21*). Total protein was determined, and water content was estimated assuming a lipid concentration equal to the protein concentration. The extracted lipids were stored in the chloroform phase at −20 °C.

Lipid extracts were analyzed on TLC using silica gel G (250 μm) 5 cm × 20 cm plates. Two solvent systems were used: system I, $CHCl_3$/MeOH/NH_4OH (65:25:5, v/v/v), for total lipids separation (*22*) and system II, $CHCl_3$/MeOH/4.2 N NH_4OH (9:7:2, v/v/v), for separation of sphingolipids (*23*). Preparative TLC for determination of PI content was done using system II.

Determination of Phospholipids and Sphingolipids. To measure total phospholipids, samples were hydrolyzed (in acid-washed tubes) in 75 μL of perchloric acid at 240 °C for 3−4 h. The acid was evaporated by heating, and

phosphate was measured as described by Valtersson and Dallner (*24*) using purified phosphatidylcholine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) as standard.

Sphingolipids were quantified by separating the total lipid extract on TLC (see above) and visualizing the lipid spots with iodine vapor. Following evaporation of the iodine, individual spots were scraped off the plate and sphingolipids were recovered from the silica gel by two consecutive extractions with 0.5 mL of methanol at 30 °C for 30 min. The two extracts were pooled and evaporated to dryness, and lipid phosphate was determined as outlined above for phospholipids.

Assays for IPC Synthase Activity. In the original IPC synthase assay using crude, untreated membranes, a substrate mix was prepared by evaporating 1.2 μL of 0.1 mg/mL C_6−NBD−ceramide in ethanol. To the dried ceramide was added 4 μL of 20 mM CHAPS and 8 μL of 50 μM PI in water. The substrate mix was then combined with 20 μg of crude *C. albicans* membranes suspended in 28 μL of 71.4 mM potassium phosphate, pH 7.0, in a microtiter plate well. Final assay volume was 40 μL, and final assay concentrations were 50 mM potassium phosphate, pH 7.0, 0.5 mg/mL membrane protein, 5 μM C_6−NBD−ceramide, 10 μM PI, and 2 mM CHAPS. The mixture was incubated at room temperature for 60 min, and the reaction was stopped by the addition of 200 μL of 96% (v/v) methanol.

The reaction product, C_6−NBD−IPC, was separated from unreacted C_6−NBD−ceramide by adsorbing onto 100 μL (sedimented gel volume) of AG4-X4 resin, formate form (*10*), in a 96-well filter plate using a vacuum manifold. The resin was washed with 5 × 200 μL of 96% (v/v) methanol, and the product was eluted with 200 μL of 1 M potassium formate in 96% (v/v) methanol. The product was quantified in a fluorescence plate reader using 466 nm exitation wavelength and measuring emission at 536 nm. Black 96-well plates were used to minimize background emission.

In the improved assay, 10 μg (protein) of CHAPS-washed membranes were preincubated with 4 nmol of PI in 28 μL of 71.4 mM potassium phosphate, pH 7.0, for 30 min in a 96-well plate. The reaction was started by addition of 12 μL of 0.1 mg/mL C_6−NBD−ceramide in ethanol or 2 mM CHAPS. Final assay volume was 40 μL, and final concentrations were 50 mM potassium phosphate, pH 7.0, 0.25 mg/mL membrane protein, 5 μM C_6−NBD−ceramide, 100 μM PI, 0.3% (v/v) ethanol, and 0.6 mM CHAPS. Following incubation at room temperature for 5−30 min (see individual experiments), the reaction was stopped by adding 200 μL of 96% (v/v) methanol. The reaction product was isolated and quantified as outlined above.

Kinetic Evaluation of Substrates and Lysophosphatidylinositol. Assays were performed essentially as described above (improved assay) with a final assay volume of 40 μL containing 50 mM potassium phosphate, pH 7.0, 10 μg of protein, and varying concentrations of PI and C_6−NBD−ceramide. Lysophosphatidylinositol (0, 25, 50, or 75 μM) was added in water, when applicable. Incubation time was 5 min at room temperature. Data were analyzed according to Segel (*25*) using the software program Prism 3.0 (GraphPad Software).

Table 1: Agents Evaluated for Solubilization of *C. albicans* Inositol Phosphorylceramide Synthase

detergent	solubilizes activity	inactivates enzyme	reconstitution w/PI	detergent type
CHAPS	N	N		zwitterionic
CHAPSO	N	N		zwitterionic
taurodeoxycholate	N	N (4%)		ionic
oleic acid	(Y)	Y	N	ionic
SDS	Y	-	-	ionic
Empigen BB	(Y)	Y	N	zwitterionic
DDMAB	(Y)	Y	N	zwitterionic
Zwittergent 3-10	(Y)	Y	N	zwitterionic
ASB-16	Y	Y	Y	zwitterionic
Triton X-100	Y	Y	Y	nonionic
Triton N-60	Y	Y	(Y)	nonionic
Nonidet P-40	Y	Y	(Y)	nonionic
octylglucoside	Y	Y	Y	nonionic
octylthioglucoside	Y	Y	(Y)	nonionic
Brij 35	N	Y	N	nonionic
Brij 58	N	Y	N	nonionic
Tween 20	N	Y	N	nonionic
Tween 80	N	Y	N	nonionic
Digitonin	(Y)	Y	N	nonionic
dodecylmaltoside	Y	-	-	nonionic
decylthiomaltoside	Y	-	-	nonionic
cyclohexyl-*n*-hexyl-maltoside	Y	-	-	nonionic
lysophosphatidylcholine	(Y)	Y	N	lysophospholipid
lysophosphatidylethanolamine	N	Y	N	lysophospholipid
lysophosphatidylglycerol	(Y)	Y	N	lysophospholipid
lysophosphatidylinositol	(Y)	Y	N (inhibitor)	lysophospholipid

RESULTS AND DISCUSSION

Solubilization of IPC Synthase−Preparation of Enzyme

An important objective of the investigation reported here was to identify conditions for an IPC synthase assay capable of generating kinetic parameters and of supporting structure−activity-relationship (SAR) studies on inhibitor compounds. Given the problems associated with using intact membranes as enzyme source (see above), attempts were first made to prepare a solubilized enzyme with retained activity. Intact, total *C. albicans* membranes were suspended in detergent-containing buffers and incubated for 1 h at 4 °C. Soluble and insoluble material was separated by centrifugation at 150 000 × *g* for 60 min, and IPC synthase activity was determined in both fractions. A number of different detergents were evaluated (summarized in Table 1).

Solubilization in Nonionic Detergents. The nonionic detergents, Triton X-100, Triton N-60, Nonidet P-40, octylglucoside, and octylthioglucoside, all appeared capable of solubilizing IPC synthase. When these detergents were used, significant portions of IPC synthase activity were recovered in the supernatant after centrifugation at 150 000 × *g* (Figure 2). Still, although these detergents appeared capable of solubilizing the enzyme, this was invariably accompanied by a drastic loss in enzyme activity. Triton X-100 extracts have been used previously in IPC synthase assays (e.g., refs *11*, *15*, and *16*), and it has been reported that inclusion of 1 mM PI in the solubilization buffer results in an increased

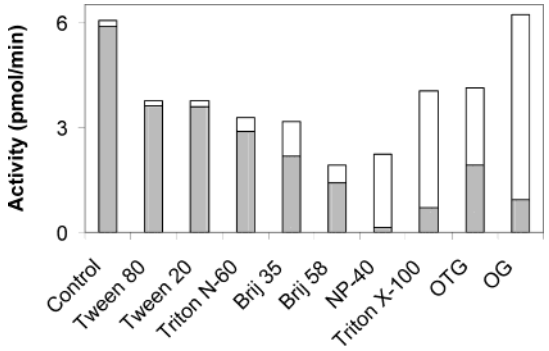

FIGURE 2: Solubilization of *C. albicans* inositol phosphorylceramide synthase in nonionic detergents**.** Crude *C. albicans* membranes were suspended in detergent-containing buffers as outlined in Materials and Methods. Following centrifugation, total IPC synthase activity was determined in the soluble (open bar) and sedimented (solid bar) fractions. OTG denotes octylthioglucoside; OG denotes octylglucoside.

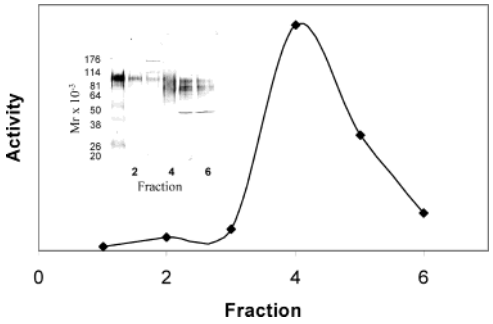

FIGURE 3: Density gradient separation of Triton X-100-extracted inositol phosphorylceramide synthase. *C. albicans* cell extracts were incubated with 1% Triton X-100 for 30 min at 4 °C. The resulting detergent extracts were fractionated on OptiPrep gradients; fractions were collected and assayed for IPC synthase activity as outlined in Materials and Methods. In the inset, OptiPrep gradient fractions were separated on a 10% Tris−glycine gel and blotted with anti-PHR1 antibodies.

recovery of activity (*11*). In our hands, inclusion of 1 mM PI increased the recovery from 2.5% to about 40% (data not shown). We also found that this number could be increased further, to about 70%, by manipulating the concentrations of protein, detergent, and PI in the detergent extract (data not shown). However, additional work reveled that although the Triton X-100-solubilized enzyme did not colocalize with intact membranes in density gradient separations, size exclusion chromatography on Sepharose 4B, Sephacryl S-200, and Sephacryl S-300 produced an apparent molecular mass (for the solubilized IPC synthase activity) of between 1.5×10^6 and 20×10^6 Da. This suggests that the extracted enzyme is not solubilized in a detergent or mixed micelle but is instead part of a large complex(s), likely composed of a number of other (lipid, protein, or both) molecules (data not shown).

Possible Association of IPC Synthase with Sphingolipid Rafts. Given the results discussed above and the fact that IPC synthase is an important component in the cellular sphingolipid biosynthesis machinery, it appeared possible the enzyme could be a raft protein. Several intrinsic mammalian and yeast membrane proteins have been found to be associated with sphingolipid rafts, densely packed aggregates of sphingolipids and sterols distributed in the more fluid environment of the phospholipid bilayers in intracellular and plasma membranes (e.g., refs *26* and *27*). Sphingolipid rafts are believed to have important functions in cell signaling and the transport and targeting of membrane components (*28*). Raft structures are commonly isolated by extraction of membrane preparations with cold Triton X-100, a detergent that appears not to disturb the densely packed lipids in the rafts. The entire lipid aggregate is instead solubilized as a complex, which can be separated from bona fide solubilized membrane components by density gradient fractionation. To investigate the possible association of IPC synthase with sphingolipid rafts, crude *C. albicans* membranes were solubilized in Triton X-100 at 4 °C and fractionated on an OptiPrep gradient, essentially as described by Bagnat et al. (*29*). Figure 3 shows that PHR1, which is a glycosylphosphatidylinositol (GPI)-anchored protein (*30*) and as such colocalized with sphingolipid rafts (*28*), was located at the top of the gradient (Figure 3, inset). By contrast, the IPC synthase activity remained in the bottom half of the gradient, consistent with the enzyme being in solution rather than associated with rafts and suggesting that the large complexes associated with Triton X-100-solubilized IPC synthase activity may constitute complexes composed of several enzyme molecules (see below) or unspecific aggregates of the enzyme with other membrane proteins, lipids, or both. Together these results suggest that Triton X-100-solubilized IPC synthase activity may be a less than ideal starting material for further enrichment of the enzyme. Moreover, the need for high levels of PI in the extraction buffer makes the crude detergent extract difficult to use as enzyme source for IPC synthase assays. Attempts to purify IPC synthase from Triton X-100 extracts have been made previously (*16*).

Although octylglucoside appears capable of solubilizing IPC synthase, inclusion of 1 mM PI (in the solubilization buffer) was again required to recover significant amounts of enzymatic activity (Figure 2). Moreover, analysis of the supernatant fraction from extraction with octylglucoside revealed that the enzyme was not truly in solution: the activity colocalized with intact membranes upon density gradient centrifugation (data not shown) and, in contrast to activity extracted with Triton X-100, the majority of the activity eluted with an apparent molecular mass larger than 20×10^6 upon size-exclusion chromatography (data not shown), suggesting a large, possibly membranous, complex(s). A small portion (<10%) of IPC synthase activity eluted in the included volume and may represent truly solubilized enzyme or enzyme in a complex(s) smaller than the 20×10^6 Da exclusion volume of the column. However, attempts to increase this portion of the activity by increasing the detergent concentration or the detergent-to-protein ratio resulted in increased losses in enzyme activity that could not be offset by inclusion of excess PI in the buffers (data not shown).

Several other nonionic detergents, in addition to Triton X-100, octylglucoside, and related compounds, were evaluated as agents for solubilization of IPC synthase. However, these detergents, which included variations of Brij and Tween, as well as the sterol-based compound digitonin, all inactivated the enzyme, and importantly, the lost activity could not be regained (or prevented) by the addition of PI (Table 1 and Figure 2). Consequently, these agents were not investigated further.

Soulublization in Lysophospholipids. A number of integral membrane proteins of both mammalian and fungal origin

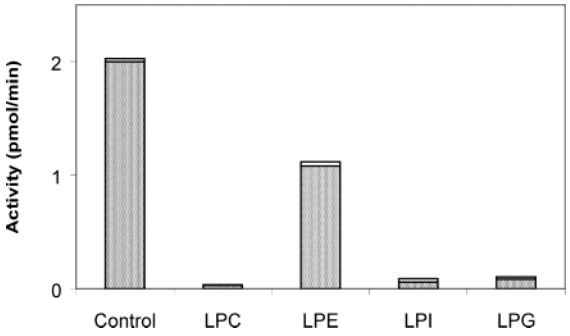

FIGURE 4: Solubilization of *C. albicans* inositol phosphorylceramide synthase in lysophospholipids. Crude *C. albicans* membranes were treated with 2% lysophospolipids. Following centrifugation at 150 000 × *g* for 60 min, the total IPC synthase activity was determined in the soluble (open bar) and sedimented (solid bar) fractions. LPC denotes lysophosphatidylcholine; LPE denotes lysophosphatidylethanol; LPI denotes lysophosphatidylinositol; LPG denotes lysophosphatidylglycerol.

have been successfully solubilized, often with retained activity, using a lysophospholipid (e.g., refs *31*−*33*). This prompted an evaluation of this type of agent for solubilization of IPC synthase. Table 1 and Figure 4 show that, while three out of the four lysophospholipids tested efficiently solubilized *C. albicans* membranes, they also equally efficiently inactivated the enzyme. The fourth agent, lysophosphatidylethanolamine, caused a less drastic reduction in enzyme activity, but this compound also did not significantly solubilize the enzyme. Interestingly, despite the strong restorative effect of intact PI on IPC synthase activity, lyso-PI inactivated the enzyme with considerable potency. This raised the possibility that the observed effect might be caused not only by lyso-PI perturbing or solubilizing the membranes but possibly also by competing with the donor substrate, PI. To investigate this, a kinetic evaluation of the inhibitory properties of lyso-PI was carried out. This revealed that, although there undoubtedly is a competitive component in the kinetics of this compound, a more significant portion of the inhibitory effect is of a noncompetitive nature. The best fit for the observed kinetics is that of a mixed mechanism of inhibition with an apparent K_i in the 50 μM range (data not shown).

The fact that significant portions of IPC synthase activity could be recovered from preparations of detergent-solubilized membranes by the addition of a reconstituting phospholipid prompted the question as to whether this effect was unique for PI or whether other phospholipids would have a similar effect. In the experiment shown in Figure 5, four different phospholipids (at a concentration of 5 mM) were evaluated for their stabilizing effect on IPC synthase activity (10 mg/mL membrane protein) during solubilization in 2% Triton X-100. The data indicate that although phosphatidylcholine (PC) and phosphatidylserine (PS) appear to have some stabilizing effect, PI is by far more efficient, even taking into account the role of PI as a substrate. Considering that the standard assay contains 100 μM PI, that is, a concentration close to the K_m for this substrate (138 μM; see below), increasing the substrate concentration to 5 mM (725 μM in the assay) should boost product formation to about twice control levels. The observed increase is 10 times higher than control levels, confirming the strong restorative effect of this phospholipid on solubilized IPC synthase.

The effect of increasing PI concentrations for the unsolubilized, membrane-associated enzyme is illustrated in Figure

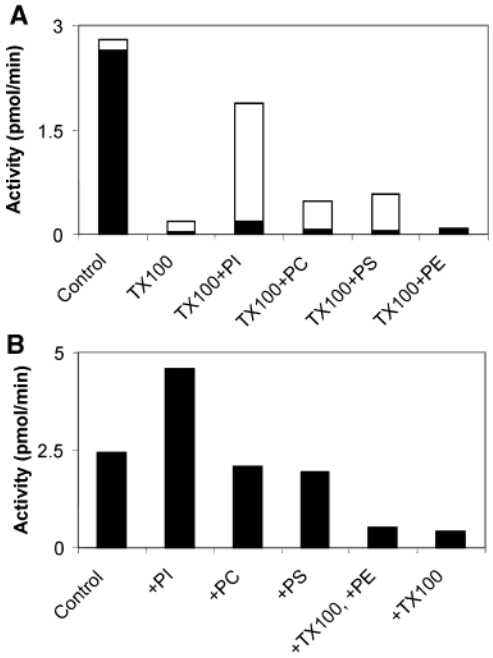

FIGURE 5: Effect of added phospholipids on the amounts of inositol phosphorylceramide synthase activity recovered from detergent-treated and native *C. albicans* membranes. In panel A, crude *C. albicans* membranes were treated with 2% Triton X-100 at 10 mg/mL membrane protein in the presence of 5 mM phospholipid. Following centrifugation at 150 000 × *g* for 90 min, the total IPC synthase activity was determined in the soluble (open bar) and sedimented (solid bar) fractions. In panel B, crude *C. albicans* membranes at 10 mg/mL membrane protein were assayed for IPC synthase activity in the presence of 5 mM phospholipid. Phosphatidylethanolamine was added in 5% Triton X-100, resulting in a final detergent concentration in the assay of 0.25%. TX100 denotes Triton X-100; PI denotes phosphatidylinositol; PC denotes phosphatidylcholine; PS denotes phosphatidylserine; PE denotes phosphatidylethanolamine.

5, panel B. As predicted, addition of 5 mM PI (725 μM in the assay) increases product formation about 100%. Addition of 5 mM PC or PS, on the other hand, seems to have, if anything, a slightly negative effect on product formation, while addition of 5 mM PE clearly appears to have an inhibitory effect. This (latter) effect, however, is likely due to the Triton X-100 required for solubilization of the PE, rather than to PE itself. As discussed above, in the absence of added PI, Triton X-100 causes significant losses in IPC synthase activity. This is again demonstrated in Figure 5, panel B, where addition of Triton X-100 alone results in 83% loss of activity. Under these conditions, the addition of PE appears to show little or no restorative effect on the enzyme, in that the loss of activity at 79% is essentially unchanged. Taken together, these results suggest that, of the phospholipids evaluated in these experiments, only PI has a significant restorative effect on IPC synthase. PI may be an absolute structural requirement for a catalytically active enzyme. Alternatively, the presence of excess substrate may stabilize (the catalytic site of) the enzyme when it is sequestered from the specific lipid environment in the Golgi membrane by the detergent.

Solubilization in Zwitterionic Detergents. Contrasting with the effect of the agents discussed above, treatment of *C. albicans* membranes with CHAPS had little effect on the activity of IPC synthase. Figure 6, panel A, shows that when intact total *C. albicans* membranes at 10 mg/mL protein were

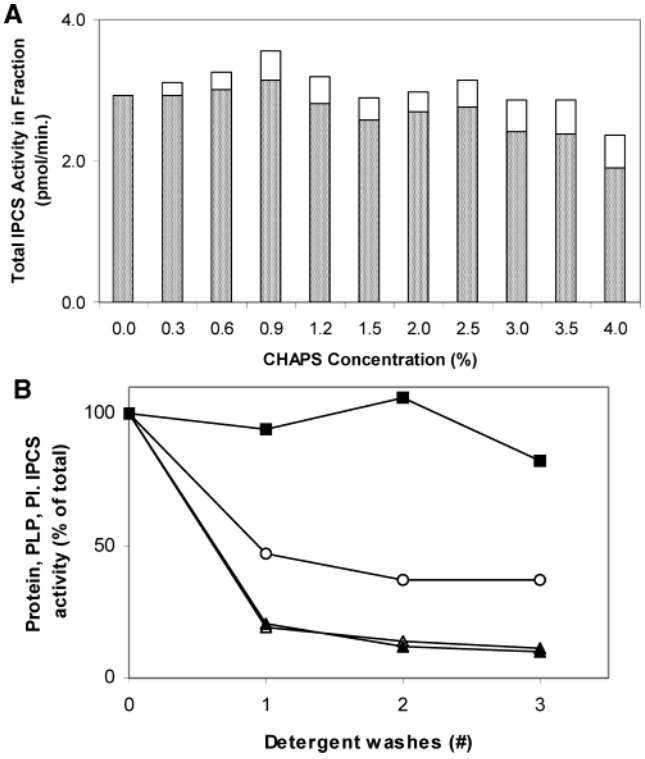

FIGURE 6: Solubilization of *C. albicans* inositol phosphorylceramide synthase in CHAPS. In panel A, crude *C. albicans* membranes were suspended in buffers containing increasing concentrations of CHAPS. Following centrifugation of the suspension, total IPC synthase activity was determined in the soluble (open bar) and sedimented (solid bar) fractions. In panel B, crude *C. albicans* membranes were treated repeatedly with buffer containing 2.5% CHAPS. Following centrifugation, total IPC synthase (IPCS) activity (■), as well as protein (○), total phospholipid (PLP; ▲), and phosphatidyl inositol (PI; △) content, was determined in the sedimented membranes from each treatment step.

exposed to increasing concentrations of CHAPS, there was little loss of activity, even at a concentration as high as 3%. Moreover, at this concentration, the majority of the IPC synthase activity is still sedimentable at 150 000 × *g*, and fractionation on Sepharose 4B revealed that the detergent-treated, sedimentable material has an apparent molecular mass exceeding 20×10^6, suggesting that it likely represents lipid-depleted membrane vesicles or large fragments thereof (data not shown). Examination of the CHAPS-treated material by electron microscopy revealed primarily membrane-like structures (data not shown). It is not clear whether the (small amounts of) enzyme activity recovered in the supernatant in these experiments actually represents IPC synthase in solution or whether it is enzyme in a (protein) complex(es) small enough or with low enough density not to sediment under the conditions used. It is noteworthy that increasing the detergent concentration from 2% to 4% has little apparent effect on the solubilization of IPC synthase and that the small portion of nonsedimentable activity remains essentially constant. To determine whether the nonsedimentable portion of enzyme could be increased by consecutive treatments with the detergent, membranes were first treated with 2.5% CHAPS at 10 mg/mL protein, as described above. Following sedimentation at 150 000 × *g* for 60 min and removal of the supernatant, the pellet was homogenized and adjusted to 10 mg/mL protein, and CHAPS was again added to 2.5%. After incubation at 4 °C for 60 min, the suspension was again

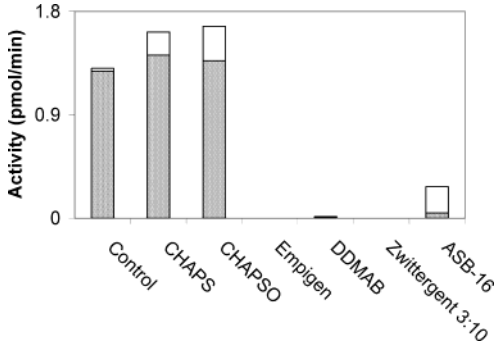

FIGURE 7: Solubilization of *C. albicans* inositol phosphorylceramide synthase with zwitterionic detergents. Crude *C. albicans* membranes were treated with 2.5% CHAPS, CHAPSO, Empigen, DDMAB, Zwittergent 3-10, or ASB-16 at 10 mg/mL membrane protein. Total IPC synthase activity in supernatant (open bar) and sedimented (solid bar) fractions was determined following separation by centrifugation at 100 000 × *g* for 60 min.

sedimented as outlined above, and the supernatant was collected. The resulting pellet was treated a third time using the same protocol, and the final pellet was suspended in buffer without detergent. Figure 6, panel B, shows that while the first extraction, removed more than 50% of the protein and 80% of the phospholipid in the membranes, subsequent extractions were less efficient. A second extraction removed an additional 10% of the protein and 5% phospholipid, bringing the total to 63% and 86%, respectively, while a third extraction had essentially no effect on the protein content but did lower the phospholipid content another 3%. Importantly, essentially no additional IPC synthase was solubilized by the second and third treatments (Figure 6, panel B), and the enzyme appeared to retain close to full activity through all three extractions.

In addition to CHAPS, five other zwitterionic detergents were evaluated for solubilization of *C. albicans* IPC synthase. Table 1 and Figure 7 show that, of these, only CHAPSO functioned similarly to CHAPS. CHAPSO extracted approximately 30% of the membrane protein with the IPC synthase remaining in the membrane fraction with no appreciable loss of activity. In contrast, the four other zwitterionic detergents both solubilized the membranes and inactivated the enzyme. Empigen and Zwittergent 3-10 completely inactivated the enzyme, while modest amounts of activity remained when DDMAB and ASB-16 were used. These results suggest that the effect of CHAPS and CHAPSO, which differ only in one hydroxyl group at the charged end of the molecule, is not related to their zwitterionic character but more likely instead to properties related to their sterol backbone. Supporting this notion is the observation that turodeoxycholate, a detergent that retains the sterol backbone of CHAPS (and CHAPSO) but has an anionic rather than zwitterionic headgroup, was found to have properties quite similar to CHAPS. The most significant difference (between the two detergents) is that a higher concentration (4%) of tDOC is required to achieve an extraction effect similar to that seen with 2.5% CHAPS (data not shown). Apparently the specific nature of the charged moieties on the two molecules is of less importance than the sterol backbone. Still, the total amount of activity recovered after extractions with tDOC consistently appeared somewhat lower, suggesting that tDOC might have a slightly increased inactivating effect on the enzyme. Taken together,

these results suggest that sterol-based detergents may interact in a unique fashion with IPC synthase. On the other hand, a major rearrangement, as compared to CHAPS, CHAPSO, and tDOC, of the hydrophilic moieties attached to the sterol backbone can apparently generate a molecule with completely different properties. Digitonin, a sterol-based agent with a pentasaccharide conjugated to the C-3 hydroxyl group and a methylated fucose-conjugated furan ring attached to carbon atom 16, has properties quite distinct from CHAPS, CHAPSO, and tDOC. Table 1 shows that this molecule efficiently solubilizes *C. albicans* membranes and also completely inactivates IPC synthase. The inactivation is not reversed by the addition of PI. These results suggest that although some modification of the sterol structure can be accommodated without changing the unique properties of the compound with respect to IPC synthase, large changes may negate these properties.

The results from the experiments discussed above suggest that IPC synthase may have properties that to some extent are unique. Two other integral membrane proteins in yeast, glucan synthase (GS) and protein mannosyltransferase 1 (PMT1), have both been solubilized in active forms using CHAPS or CHAPS/DOC combinations (e.g., refs *34*−*38*). The domain structures of GS and PMT1 appear similar to IPC synthase. Both contain several transmembrane domains, and at least the topology of PMT1 is believed to be the same as that of IPC synthase with the active site localized on the lumenal side of the membrane. Both GS and PMT1 are solubilized at CHAPS concentrations well below 4%. However, while IPC synthase reportedly is localized in the Golgi complex (*14*), GS is localized in plasma membrane and PMT1 is believed to be localized in the endoplasmic reticulum (*39*). It is possible that the specific lipid composition of the Golgi membrane is less susceptible to disruption by detergents such as CHAPS. Alternatively, the resistance of IPC synthase to solubilization with CHAPS is related to specific properties of the enzyme itself. The *C. albicans* IPC synthase sequence includes a motif close to the C-terminus comprising nine consecutive glutamic and aspartic acid residues, as well as a six amino acid cluster of positively charged amino acids near the N-terminal end of the protein. It has been reported that such sequences frequently are involved in protein−protein interactions (e.g., refs *40*−*42*). Hence it appears possible that IPC synthase, in situ, exists in a complex with either other IPC synthase molecules or other proteins and that this may be a contributing factor to the poor solubility of the molecule.

Development of a Novel IPC Synthase Assay

The results discussed above suggest that IPC synthase may be difficult to extract from the membrane in active form. However, since the enzymatic activity is largely unaffected by removal of more than half of the protein and almost 90% of the lipids in the crude membrane fraction, it appeared possible that a reasonably capable IPC synthase assay could be developed using the enriched, substrate-depleted enzyme present in CHAPS-treated membranes. Figure 8 shows a comparison of IPC synthase product formation over time using crude native *C. albicans* membranes and membranes washed with 2.5% CHAPS. The graph in panel A clearly demonstrates that the assay using crude membranes is only to a limited extent dependent on the addition of the donor

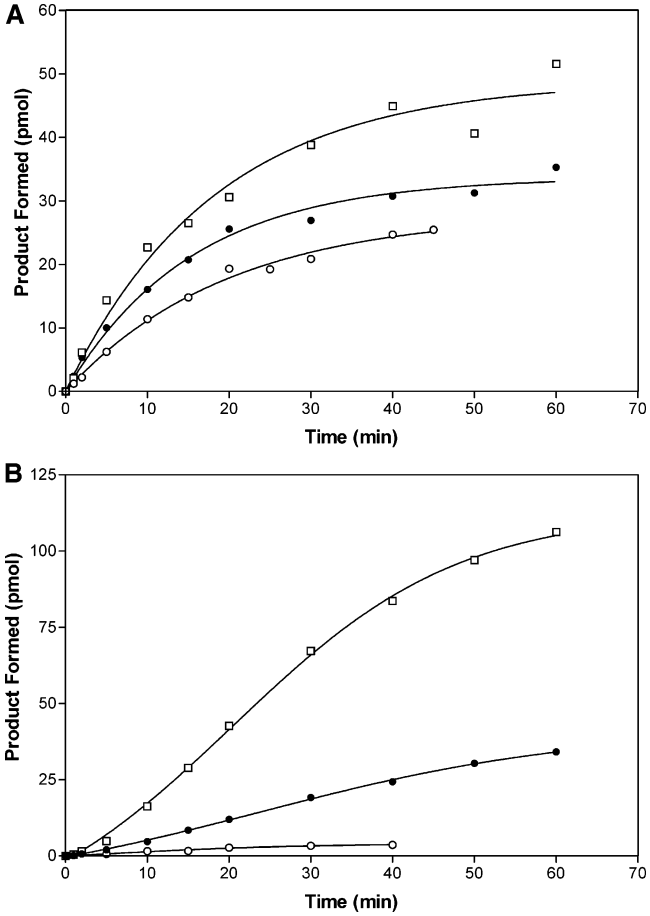

FIGURE 8: Assays for inositol phosphorylceramide synhthase activity using native and detergent-treated *C. albicans* membranes. IPC synthase activity was assayed using native (panel A) and detergent-treated (panel B) membranes as outlined in Material and Methods in the presence of 0 (○), 10 (●), or 50 (□) μM added donor substrate (PI).

substrate, suggesting that considerable amounts of PI are present in the preparation. Previous work has shown that approximately 20% of the phospholipid content in *S. cerevisiae* Golgi membranes is PI. Moreover, measurements of the overall PI content in *C. albicans* is consistent with similar levels in this organism (*17*, *43*, *44*). Consistent with this, significant amounts of product are formed without any addition of PI, and inclusion of 50 μM PI in the assay resulted in a less than 2-fold increase in product formation. It is also clear that product formation is not linear but instead tapers off with time. Additional experiments showed that this is not due to exhaustion of either of the substrates in that addition of more PI or ceramide at the onset or 30 min into the assay had virtually no effect on product formation. However addition of more enzyme (membranes) did result in a resumed generation of product (data not shown). Since other experiments demonstrated that incubation of the enzyme (without substrates) under assay conditions does not cause any measurable loss of activity (data not shown), a likely explanation for the loss of product formation in these assays is product inhibition or back-up of newly formed product in the membrane vesicles. Panel B in Figure 8 shows product formation using membranes washed with 2.5% CHAPS. In contrast to the assay using intact membranes and consistent with the removal of almost 90% of the endogenous PI in the preparation (Figure 6, panel B), this assay is almost

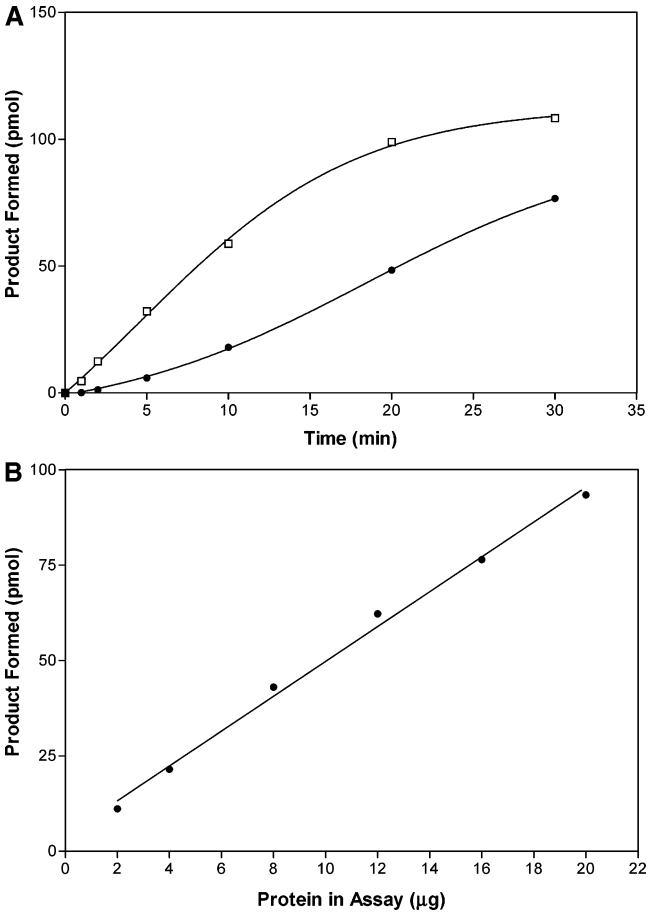

FIGURE 9: Effect of substrate preincubation and enzyme concentration on the inositol phosphorylceramide synthase activity in detergent-washed *C. albicans* membranes. IPC synthase assays were carried out using CHAPS-treated *C. albicans* membranes. Panel A presents the results of assays with (●) no preincubation with the donor substrate and (○) enzyme preincubated in 4 nmol of PI. Panel B shows the results of assays carried out using increasing amounts of CHAPS-treated membranes preincubated with PI.

entirely dependent on the addition of PI. The background activity without added PI is less than 5% of the activity observed in the presence of added substrate. Moreover, following a short lag period, product formation is linear until exhaustion of substrate. More than 50% of the ceramide acceptor in the assay is consumed at 45 min. The lag at the beginning of the time curve suggested a rate-limiting event upstream of the enzymatic transfer. Since the substrates for IPC synthase in situ likely are delivered to the enzyme in the membrane and since the washed membranes used in this assay are depleted in lipids, we considered the possibility that the lag period might be related to a slower diffusion of PI into the depleted membranes or onto the active site of the enzyme. The data shown in Figure 9, panel A, support this hypothesis. Preincubation of the enzyme preparation with PI boosts early product formation more than 5 times and essentially eliminates the lag. Under these conditions, the reaction is essentially linear until exhaustion of substrate(s). Further experimentation revealed that an additional approximately 80−100% boost in efficiency could be realized by delivering the acceptor substrate to the assay dissolved in ethanol instead of in a detergent solution (data not shown). Addition of increasing amounts of detergent-washed membranes to the assay demonstrated that product formation was linear with enzyme concentration up to at least 0.5 mg/mL,

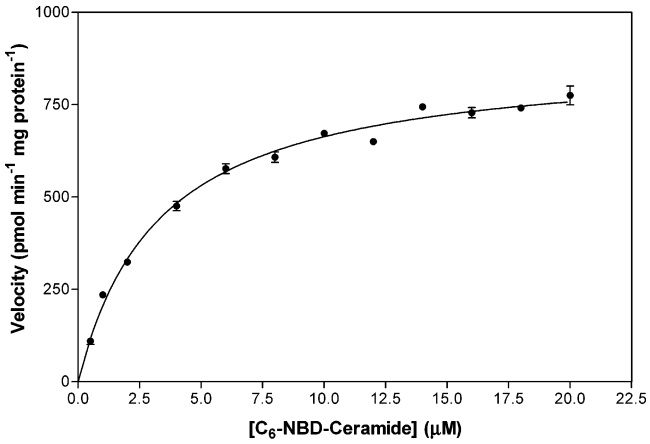

FIGURE 10: Determination of kinetic parameters for the acceptor substrate C6−NBD−ceramide. Assay conditions were as described in Materials and Methods using preincubated, CHAPS-treated *C. albicans* membranes and saturating concentrations of donor substrate. The vales are given as means ± SE ($n = 3$).

that is, twice the concentration normally used in the assay (Figure 9, panel B).

Using the assay conditions described above, we determined the kinetic parameters for the acceptor substrate, C_6−NBD−ceramide, and the donor substrate, PI. The apparent K_m for C_6−NBD−ceramide using detergent-washed *C. albicans* membranes is approximately 3.3 μM (Figure 10). This is in agreement with a previous estimate (*12*). The apparent V_{max} was 884 pmol min^{-1} mg^{-1} of protein. Determination of a K_m value for PI was less straightforward. Analysis of the data using standard Michaelis−Menten kinetics, yielded a less than perfect curve fit (Figure 11, panel A). The poor fit was primarily due to a drop in velocity (to below V_{max}) at high substrate concentrations (Figure 11, panels A and B). This was a consistent finding in several experiments and led to the conclusion that high PI concentrations may be inhibitory for IPC synthase. Reanalysis of the assay data using an algorithm that incorporates a substrate inhibition parameter confirmed this conclusion and yielded an almost perfect curve fit (Figure 11, panel B). The apparent K_m obtained by this analysis was 138 μM, that is, considerably higher than the K_m for the ceramide. The apparent V_{max} was 1864 pmol min^{-1} mg^{-1} of protein. To our knowledge, kinetic parameters for this substrate have not been reported previously. The relatively low affinity of PI for the IPC synthase enzyme is consistent with the high levels of this substrate in the intact yeast Golgi membrane (*17*). It has been established previously that phytoceramide is the rate-limiting substrate in the in situ IPC synthase reaction and that the condensation of palmitoyl-CoA with serine, the initial reaction in sphingolipid biosynthesis, is the rate-limiting step in the entire biosynthetic pathway (*45*). The data reported here are in excellent agreement with these observations.

In conclusion, *C. albicans* IPC synthase appears to be an enzyme with a strict dependence on the specific lipid environment in its native (Golgi) location. It is readily inactivated by most membrane-perturbing agents, and it is difficult to solubilize with retained enzymatic activity. Although enzymatic activity can be restored in certain detergent extracts, this appears to be associated with incomplete solubilization or the enzyme existing in large complexes. In addition, IPC synthase does not appear to be a

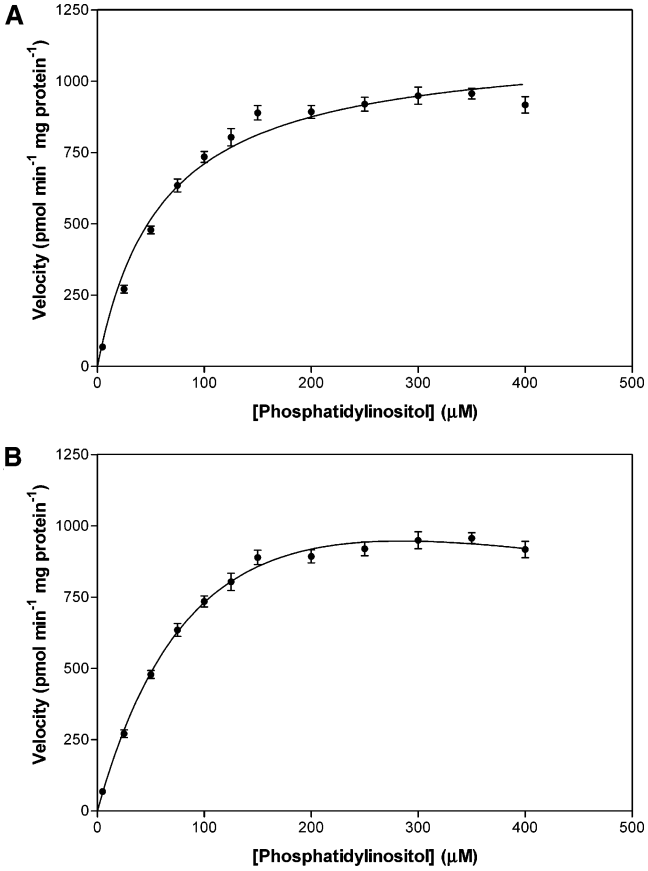

FIGURE 11: Determination of kinetic parameters for the donor substrate phosphatidylinositol. Assay conditions were as described in Materials and Methods. Preincubated, CHAPS-treated *C. albicans* membranes were used with increasing amounts of PI and saturating concentrations of acceptor substrate. Panels A and B show the data fitted to Michaelis−Menten and substrate-inhibition kinetics, respectively. The values are given as means ± SE ($n = 3$).

raft protein. Certain sterol-based detergents, despite their considerable effect on overall membrane structure and integrity, appear less prone to inactivate the enzyme. This has allowed the developed an IPC synthase assay that is substrate-dependent, linear, and reproducible. The assay does not require a recombinant enzyme or purification of the native enzyme. It is carried out in a 96-well format and is adaptable to large-scale screening efforts. Importantly, the assay is capable of generating kinetic parameters for both substrates, and preliminary studies suggest that it is readily applicable to the evaluation of (putative) IPC synthase inhibitors and to SAR studies of such compounds.

REFERENCES

1. Smith, S. W., and Lester, R. L. (1974) Inositol phosphorylceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate, *J. Biol. Chem. 249*, 3395−3405.
2. Dickson, R. C., and Lester, R. L. (1999) Yeast sphingolipids, *Biochim. Biophys. Acta 1426*, 347−357.
3. Lester, R. L., and Dickson, R. C. (1993) Sphingolipids with inositolphosphate-containing headgroups, *Adv. Lipid Res. 26*, 253−274.
4. Warnecke, D., and Heinz, E. (2003) Recently discovered functions of glucosylceramides in plants and fungi, *Cell. Mol. Life Sci. 60*, 919−941.
5. Patton, J. L., and Lester, R. L. (1991) The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane, *J. Bacteriol. 173*, 3101−3108.
6. Hechtberger, P., Zinser, E., Saf, R., Hummel, K., Paltauf, F., and Daum, G. (1994) Characterization, quantification and subcellular localization of inositol-containing sphingolipids of the yeast, *Saccharomyces cerevisiae*, *Eur. J. Biochem. 225*, 641−649.
7. Vincent, V. L., and Klig, L. S. (1995) Unusual effect of myo-inositol on phospholipid biosynthesis in *Cryptococcus neoformans*, *Microbiology 141*, 1829−1837.
8. Schneiter, R. (1999) Bravo little yeast, please guide us to Thebes: Sphingolipid function in *S. cerevisiae*, *BioEssays 21*, 1004−1010.
9. Nagiec, M. M., Nagiec, E. E., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1997) Sphingolipid synthesis as a target for antifungal drugs, *J. Biol. Chem. 272*, 9809−9817.
10. Mandala, S. M., Thornton, R. A., Rosenbach, M., Milligan, J., Garcia-Calvo, M., Bull, H. G., and Kurtz, M. B. (1997) Khafrefungin, a novel inhibitor of sphingolipid synthesis, *J. Biol. Chem. 272*, 32709−32714.
11. Mandala, S. M., Thornton, R. A., Milligan, J., Rosenbach, M., Garcia-Calvo, M., Bull, H. G., Harris, G., Abruzzo, G. K., Flattery, A. M., Gill, C. J., Bartizal, K., Dreikorn, S., and Kurtz, M. B. (1998) Rustmicin, a potent antifungal agent, inhibits sphingolipid synthesis at inositol phosphoceramide synthase, *J. Biol. Chem. 273*, 14942−14949.
12. Zhong, W., Murphy, D. J., and Georgopapadakou, N. H. (1999) Inhibition of yeast inisitol phosphorylceramide synthase by aureobasidin A measured by a fluorometric assay, *FEBS Lett. 463*, 241−244.
13. Kurome, T., and Takesako, K. (2000) SAR and potential of the aureobasidin class of antifungal agents, *Curr. Opin. Anti-Infect. Invest. Drugs 2*, 375−386.
14. Levine, T. P., Wiggins, C. A. R., and Munro, S. (2000) Inositol phosphorylceramide synthase is located in the Golgi apparatus of *Saccharomyces cerevisiae*, *Mol. Biol. Cell 11*, 2267−2281.
15. Ko, J., Cheah, S., and Fischl, A. S. (1995) Solubilization and characterization of microsomal-associated phosphatidylinositiol: ceramide phosphoinositol transferase from *Saccaromyces cerevisiae*, *J. Food Biochem. 19*, 253−267.
16. Fischl, A. S., Liu, Y., Browdy, A., and Cremesti, A. E. (1999) Inositolphosphoryl ceramide synthase from yeast, *Methods Enzymol. 311*, 123−130.
17. Leber, A., Hrastnik, C., and Daum, G. (1995) Phospholipid-synthesizing enzymes in Golgi membranes of the yeast *Saccharomyces cerevisiae*, *FEBS Lett. 337*, 271−274.
18. Hendershot, L. L., Aeed, P. A., Kezdy, F. J., and Elhammer, Å. P. (2002) An efficient assay for dolichyl phosphate-mannose: protein O-mannosyltransferase, *Anal. Biochem. 307*, 273−279.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
20. Wessel, D.,, and Flügge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, *Anal. Biochem. 138*, 141−143.
21. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipd extraction and purification, *Can. J. Biochem. Physiol. 37*, 911−917.
22. Lavie, Y., Cao, H., Bursten, S. L., Giuliano, A. E., and Cabot, M. C. (1996) Accumulation of glucosylceramides in multidrug-resistant cancer cells, *J. Biol. Chem. 271*, 19530−19536.
23. Wells, G. B., Dickson, R. C., and Lester, R. L. (1996) Isolation and composition of inositolphosphorylceramide-type sphingolipids of hyphal forms of *Candida albicans*, *J. Bacteriol. 178*, 6223−6226.
24. Valterson, C., and Dallner, G. (1982) Compartmentalization of phophatidylethanolamine in microsomal membranes from rat liver, *J. Lipid Res. 23*, 868−876.
25. Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley and Sons, Inc., New York.
26. Simons K., and Ikonen E. (1997) Functional rafts in cell membranes. *Nature 387*, 569−572.
27. Brown D. A., and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts, *J. Biol. Chem. 275*, 17221−17224.
28. Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A., and Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast, *Proc. Natl. Acad. Sci. U.S.A. 97*, 3254−3259.

29. Bagnat, M., Chang, A., and Simons, K. (2001) Plasma membrane protein ATPase Pma1p requires raft association for surface delivery in yeast, *Mol. Biol. Cell 12*, 4129−4138.
30. Fonzi, W. A. (1999) *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans, *J. Bacteriol 181*, 7070−7079.
31. Mao, Q., and Scarborough, G. A. (1997) Purification of functional human P-glycoprotein expressed in *Saccharomyces cerevisiae*, *Biochim. Biophys. Acta 1327*, 107−118.
32. Huang, P., Liu, Q., and Scarborough, G. A. (1998) Lysophosphatidylglycerol: a novel effective detergent for solubilizing and purifying the cystic fibrosis transmembrane conductance regulator, *Anal. Biochem. 259*, 89−97.
33. Kern, R., Joseleau-Petit, D., Chattopadhyay, M. K., and Richarme, G. (2001) Chaperone-like properties of lysophospholipids, *Biochem. Biophys. Res. Commun. 289*, 1268−1274.
34. Strahl-Bolsinger, S., and Tanner, W. (1991) Protein O-glycosylation in *Saccharomyces cerevisiae*. Purification and characterization of the dolichyl-phosphate-D-mannose-protein O-D-mannosyltransferase, *Eur. J. Biochem. 196*, 185−190.
35. Mol, P. C., Park, H.-M., Mullins, J. T., and Cabib, E. (1994) A GTP-binding protein regulates the activity of (1→3)-beta-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis, *J. Biol. Chem. 269*, 31267−31274.
36. Dotson, S. B., Rush, J. S., Ricketts, A. D., and Waechter, C. J. (1995) Mannosylphosphoryldolichol-mediated O-mannosylation of yeast glycoproteins: stereospecificity and recognition of the alpha-isoprene unit by a purified mannosyltransferase, *Arch. Biochem. Biophys. 316*, 773−779.
37. Inoue, S. B., Takewaki, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y., and Watanabe, T. (1995) Characterization and gene cloning of 1,3-beta-D-glucan synthase from *Saccharomyces cerevisiae*, *Eur. J. Biochem. 231*, 845−854.
38. Frost, D., Brandt, K., Estill, C., and Goldman, R. (1997) Partial purification of (1,3)-beta-glucan synthase from *Candida albicans*, *FEMS Microbiol. Lett. 146*, 255−261.
39. Strahl-Bolsinger, S., and Scheinost, A. (1999) Transmembrane topology of pmt1p, a member of an evolutionarily conserved family of protein O-mannosyltransferases, *J. Biol. Chem. 274*, 9068−9075.
40. Nagai, A., Saijo, M., Kuraoka, I., Matsuda, T., Kodo, N., Nakatsu, Y., Mimaki, T., Mino, M., Biggerstaff, M., Wood., R. D., Sijbers, A., Hoeijmakers, J. H. J., and Tanaka, K. (1995) Enhancement of damage-specific DNA binding of XPA by interaction with the ERCC1 DNA repair protein, *Biochem,. Biophys. Res. Commun. 211*, 960−966.
41. Hartmann, A. M., Nayler, O., Schwaiger, F. W., Obermeier, A., and Stamm, S. (1999) The interaction and colocalization of Sam68 with the splicing-associated factor YT521-B in nuclear dots is regulated by the Src family kinase p59(fyn), *Mol. Biol. Cell 10*, 3909−3926.
42. Poetsch, A., Molday, L. L., and Molday, R. S. (2001) The cGMP-gated channel and related glutamic acid-rich proteins interact with peripherin-2 at the rim region of rod photoreceptor disc membranes, *J. Biol. Chem. 276*, 48009−48016.
43. Hitchcock, C. A., Barrett-Bee, K. J., and Russel, N. J. (1986) The lipid composition of azole-sensitive and azole-resistant strains of *Candida albicans*, *J. Gen. Microbiol. 132*, 2421−2431.
44. Hitchcock, C. A., Barrett-Bee, K. J., and Russel, N. J. (1989) The lipid composition and permeability to the triazole antifungal antibiotic ICI 153066 of serum-grown mycelial cultures of *Candida albicans*, *J. Gen. Microbiol. 135*, 1949−1955.
45. Dickson, R. C. (1998) Sphingolipid functions in *Saccharomyces cerevisiae*: comparison to mammals, *Annu. Rev. Biochem. 67*, 27−48.

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