Sphingolipid Synthesis as a Target for Antifungal Drugs

COMPLEMENTATION OF THE INOSITOL PHOSPHORYLCERAMIDE SYNTHASE DEFECT IN A MUTANT STRAIN OF SACCHAROMYCES CEREVISIAE BY THE AUR1 GENE

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We have identified a Saccharomyces cerevisiae gene necessary for the step in sphingolipid synthesis in which inositol phosphate is added to ceramide to form inositol-P-ceramide, a reaction catalyzed by phosphati-
dylinositol-ceramide phosphoinositol transferase (IPC synthase). This step should be an effective target for antifungal drugs. A key element in our experiments was the development of a procedure for isolating mutants defective in steps in sphingolipid synthesis downstream from the first step including a mutant defective in IPC synthase. An IPC synthase defect is supported by data showing a failure of the mutant strain to incorporate radioactive inositol or N-acetylphosphinganine into sphingolipids and, by using an improved assay, a demonstration that the mutant strain lacks enzyme activity. Furthermore, the mutant accumulates ceramide when fed exogenous phytosphingosine as expected for a strain lacking IPC synthase activity. Ceramide accumulation is accompanied by cell death, suggesting the presence of a ceramide-activated death response in yeast. A gene, AUR1 (YKL004w), that complements the IPC synthase defect and restores enzyme activity and sphingolipid synthesis was isolated. Mutations in AUR1 had been shown previously to give resistance to the antifungal drug aureobasidin A, leading us to predict that the drug should inhibit IPC synthase activity. Our data show that the drug is a potent inhibitor of IPC synthase with an IC_{50} of about 0.2 nM. Fungal pathogens are an increasing threat to human health. Now that IPC synthase has been shown to contain it as well since it has been found in all fungi studied to date (1).

The sphingolipid biosynthetic pathway of S. cerevisiae is diagrammed in Fig. 1. Work presented in this paper focuses on the modification of the 1-hydroxyl of phytoceramide by the addition of myo-inositol phosphate to form IPC which is then mannosylated to yield mannosyl inositol-P-ceramide (MIPC). The final step in S. cerevisiae sphingolipid synthesis is the addition of inositol-P to MIPC to yield the major sphingolipid mannose-(inositol-P)_{2}-ceramide (M(IP)_{2}C (3–5)). The later steps in sphingolipid synthesis in S. cerevisiae (Fig. 1) are tentative because none of the enzymes have been purified, the reaction requirements are poorly defined, and the stoichiometry has not been determined (reviewed in Ref. 1).

We previously searched for mutants defective in sphingolipid synthesis by screening for cells requiring exogenous sphingoid long chain base for growth (Lcb^{−} phenotype). Approximately 50 Lcb^{−} mutants fell into only two complementation groups, designated LCB1 and LCB2 (6, 7), both of which are necessary for serine palmitoyltransferase activity and are thought to encode subunits of the enzyme (8). No mutants were found for the next step, 3-ketosphinganine reductase, possibly because of accumulation of lethal concentrations of 3-ketosphinganine. Mutants defective in the later steps in sphingolipid synthesis, ceramide synthesis, and beyond, would not have been recovered because neither exogenous ceramide nor sphingolipids containing phosphoinositol were able to support growth of Lcb^{−} mutants (7). In addition, accumulation of ceramide should have inhibited growth since exogenous N-acetylceramide does inhibit growth

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1 The abbreviations used are: IPC, inositol phosphorylceramide; AbA, aureobasidin A; AUR1, a gene that can mutate to give resistance to AbA; Aur1p, the protein encoded by AUR1; IPC-3, IPC whose ceramide is N-hydroxyfattyacylphytosphingosine; Lcb, long chain base; Lcb1, the Lcb1 subunit of serine palmitoyltransferase; LCB1, a gene encoding Lcb1; Lcb2, the Lcb2 subunit of serine palmitoyltransferase; LCB2, a gene encoding Lcb2; MIPC, mannosyl inositol-P-ceramide; M(IP)_{2}C, mannosyl-(inositol-P)_{2}-ceramide; PHS, phytosphingosine; PI, phosphati-
dylinositol; SLC, sphingolipid compensatory; CHAPS, 3-[3-cholami-
dopropyl][dimethylammonio]-1-propanesulfonic acid; PYED, complex medium.
IPC Synthase

of *S. cerevisiae* cells (9, 10).

In the experimental approach described here for isolating strains defective in the later steps in sphingolipid biosynthesis, the potential problems of growth inhibition and killing by the build up of a sphingolipid intermediate were circumvented by the potential problems of growth inhibition and killing by the of sphingolipids essential for vegetative growth (13). The of exogenous long chain base, to make novel glycerophospho-

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**FIG. 1.** Sphingolipid biosynthetic pathway in *S. cerevisiae*. Known pathway intermediates, substrates, genes (LCB1 and LCB2), and enzyme are indicated (1). Undefined steps are indicated in brackets or by question marks.

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Experiments with drug resistance in *S. cerevisiae* had shown that mutations in AUR1 (14, 15) confer resistance to the antifungal drug aureobasidin A (AbA), a cyclic depsipeptide produced by *Aureobasidium pullulans* R106. Because the function of the Aur1 protein was not known it was not clear why AbA was fungicidal. We show here that AbA is a potent inhibitor of IPC synthase and offer an explanation for its fungicidal activity.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Culture Conditions—** *S. cerevisiae* strains are: 7R6 (MATa ura3-52 leu2-3, 112 lcb1::URA3 SLC1-1 ade1, Ref. 12) which was derived from wild type strain SJ218 (MATa ura3-52 leu2-3, 112 ade1); YPH2 (MATa ura3-52 lys2-80 amber ade2−10, Ref. 16); AG27-61 (MATa ura3-52 leu2-3, 112 lcb1::URA3 SLC1-1 lys2-80 amber ade1 ipc1-I) was derived from 7R6 by the procedure described below. A diploid version of strain AG27-61 (termed AGD27-61) was made by transforming haploid cells with a plasmid (pHO-12, Ref. 17) carrying the HO endonuclease gene, responsible for switching of the mating type (18). Leu² transformants were streaked onto PYED plates and colonies containing MATa/MATa diploid cells were identified by their large, ellipsoidal morphology. Diploids were screened on defined medium lacking leucine for Leu− cells, indicating loss of the plasmid carrying the HO gene, and tested for transformation efficiency using pRS315 (LEU2 CEN4, Ref. 16).

Construction of pLCB1-5 began by inserting the *S. cerevisiae* ADE2 gene as a PstI-SpeI DNA fragment into pRS315 cut with the same restriction endonucleases. The resulting plasmid was cut with Nael and SacI and ligated to the LCB1 gene obtained from pTZ18-LCB1 (19) as an NruI-SalI DNA fragment. pIPC1 was isolated from the recombinant DNA library described below in which the 4307-base pair insert corresponds to *S. cerevisiae* Chromosome XI between coordinates 432,813 and 437,119 as described in the Saccharomyces cerevisiae genome data base at Stanford University (http://genome-www.stanford.edu).

A recombinant DNA library containing about 160,000 plasmids, 95% of which carried an insert, was constructed. Genomic DNA from derivatives of strain 4R3 resistant to pH 4.1 was isolated, pooled, and 10 μg was partially digested with *Sau*3AI. DNA fragments of 5–10 kilobases were isolated from an agarose gel and ligated with about 1 μg of *BamHI*-digested, alkaline phosphatase-treated pRS315. Ligated DNA was purified using GeneClean (Bio-101, La Jolla, CA), electroporated into Escherichia coli XL1-Blue cells (Stratagene, La Jolla, CA), and plasmid DNA was prepared from ampicillin-resistant colonies selected on Petri plates.

Yeast were grown on modified PYED (buffered to pH 5.0, PYED-5.0) which contained 1% yeast extract (Difco), 2% Bacto-Peptone (Difco), 2 or 4% glucose, 50 mM sodium succinate (pH 5.0), inositol (50 mg/liter), and potassium phosphate monobasic (0.5 g/liter) (19), or on defined medium supplemented as described (19) and containing, when necessary, 25 μM phosphotungstate (PHS). PYED buffered to pH 4.1 (PYED-4.1) was made by mixing 290 ml of autoclaved yeast extract (1%) plus peptone (2%), 100 ml of filter sterilized glycollcine (0.5 μM, pH 3.1), 200 ml of glucose (20%), 10 ml of inositol (0.5%), and 100 ml of potassium phosphate monobasic (0.5%). In a previous publication (20) we erroneously referred to this medium as having a pH of 3, but the actual pH is 4.1. For some experiments indicated under “Results,” the agar in Petri plates was replaced with agarose (Fisher, BP160-500).

**Isolation of Strain AG27—** Strain 7R6 was grown overnight in PYED plus 25 μM PHS (referred to here as the medium) and then mutagenized with ethylmethanesulfonate to give 20% killing (7). Mutagenized cells were diluted to an absorbance of 0.60 at 600 nm (λ600) of 0.4 with medium, incubated with shaking at 30 °C for 7 h, during which time the A600 increased to 2.5, centrifuged, resuspended in 2 ml of medium, and sonicated using a microtip (Heat Systems-Ultrasonic) for 2 min to disrupt clumped cells. One ml of cells was layered on 4 ml of 30% sodium diatrizoate (7) and centrifuged at 10 °C in a Sorvall RT6000B centrifuge for 4 min at 2000 rpm. Most cells were at the interface but a faint pellet of dense cells was present at the bottom of the tube. The liquid was aspirated and the cell pellet was carefully resuspended in 0.5 ml of medium to avoid mixing with cells stuck to the side of the tube. Resuspended pellets from two tubes were mixed and re-centrifuged on 30% sodium diatrizoate. The cell pellet was resuspended in medium and about 500 cells were spread on PYED plates containing 25 μM PHS.

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2 M. S. Skrzypek, R. L. Lester, and R. C. Dickson, unpublished data.
Two days later only about 15 colonies per plate were visible, suggesting only 3% of the dense cells were viable.

**Inositol Labeling of Sphingolipids in Vivo—**Sphingolipid synthesis was measured by growing cells to saturation at 30 °C in PYED, diluting to an A600 of 0.1 with fresh PYED medium containing 10 μg/ml inositol and 250 μCi/ml of [3H]inosine (Du Pont NEN), and grown overnight to saturation at 30 °C. Cells were washed with 25 ml of 0.1 M sodium phosphate (pH 5.5) by centrifugation and resuspended in the same buffer at 50 A600 units/ml. A reaction containing 0.4 ml of 0.5% Tergitol, 0.4 ml of 0.5 M sodium succinate (pH 5.5), 0.1 ml of 40% glucose, and 10 μl of 2.5 mM H5S in 95% ethanol was initiated by addition of 0.2 ml of the labeled cells. At time 0, a 0.2-ml sample and at 1.5 h a 0.5-ml sample of labeled cells were treated with trichloroacetic acid at a final concentration of 5% for 20 min at 0 °C. Cells were centrifuged, washed 3 times with 1 ml of 5% trichloroacetic acid, and finally with water. Cell pellets were extracted with 1 ml of 95% ethanol:water:diethyl ether:pyridine:concentrated NH4OH (15:15:5:1:0.018, v/v) for 60 min at 60 °C (21). After centrifuging while warm, a 0.5-ml sample of the supernatant fluid was evaporated under a stream of N2, dissolved in 1 ml of monomethylamine reagent, and heated for 30 min at 52 °C to decylate the acylster sphingolipids (22). The sample was dried and dissolved in 0.5 ml of chloroform:methanol:water (16:18:5). Qualitative analysis of 25-ml samples was carried out by silica gel thin layer chromatography on 20-cm Whatman LK5 plates developed with chloroform, methanol, 4.2 x NH4OH (9:7:2). Radioactivity was measured by using a BioScan apparatus.

**[3H]Acetylphosphinganine Labeling of Sphingolipids in Vivo—**Cellular conversion of [3H]acetylphosphinganine to IPC was measured as follows. PYED medium was added to tubes containing solid [3H]acetylphosphinganine (860 cpm/μmol) and the mixture was dissolved by treatment for 5 min in an ultrasonic bath. Log phase cells, grown in PYED medium, were centrifuged, suspended in fresh medium and added to the radiolabeled medium to give a final cell density of 1.5 A600 units/ml. The final concentration of N-acetylphinganine was 5.0 μM. At the indicated times, 1-ml samples were removed, quenched with trichloroacetic acid, and processed as in the inositol-labeling procedure.

Qualitative analysis of 25-ml samples was carried out on 20-cm Whatman HP-K plates developed with chloroform, methanol, 4.2 x NH4OH (9:7:2). Each lane contained 2 nmol of IPC-3 (IPC with phytosphingosine and a monohydroxylated fatty acid, Ref. 1) internal standard. The sample was dried, dissolved in 0.5 ml of 2.5 M PHS in 95% ethanol was initiated by addition of 0.1 M sodium phosphate (pH 5.5) by centrifugation and resuspended in the same buffer at an A600 of 0.2 in fresh medium containing 25 μM ethanol. Cells were incubated at 30 °C with shaking. At 0, 2, and 4 h 100 A600 units were treated with a final concentration of 5% (v/v) trichloroacetic acid, washed several times by centrifugation with water, and the pellet was extracted with 5 ml of chloroform:methanol (1:1) for 30 min at 50 °C. The extract was dried and resuspended in 0.5 ml of chloroform and applied to a 1-ml column of Adsorbosil 100–200 mesh (Applied Sciences Inc.) in a Pasteur pipette packed in methanol and equilibrated with chloroform. After washing with 3 ml of chloroform, ceramides were eluted with 3 ml of chloroform:methanol (9:1) and the eluates were dried. Samples as well as 5–20 nmol of ceramide 3 standard were dissolved in absolute ethanol and dried to remove traces of water. Perbenzoylation was achieved by treating each sample with 0.5 ml of benzoyl chloride: pyridine (1.9, v/v) (anhydrous, Aldrich) for 2 h at 70 °C followed by addition of 1 ml of methanol and heating for 30 min at 70 °C. After solvent removal, 1 ml of saturated sodium carbonate in methanol plus 50 μg of solid sodium carbonate were added followed by extraction three times with 1 ml of hexane. The combined hexane extracts were evaporated to dryness and dissolved in 1.0 ml of hexane which was applied to a 1.0-ml column (in Pasteur pipette) of AG4-X4: acetate form, packed in water, equilibrated with absolute ethanol, then hexane. After elution with 3 ml of hexane, the eluates were dried and dissolved in 200 μl of hexane.

The benzoylated ceramide (25 μl) was separated and quantified by chromatography on a 0.45 × 30-cm, 5-μm Lichrosorb Si 60 column, eluted with hexane:di ethanolamine (95:5) at a flow rate of 1.0 ml/min monitored at 228 nm. Data were integrated by using Millennium software (Waters and Co.).

**Preparation of {[4,5}-{3H]N-Acetylphosphinganine—**N-Acetylphosphingosine (Matreya, Inc.) was reduced in ethanol with tritium gas (American Radiolabeled Chemicals, Inc.) in the presence of Adams catalyst. The sample was dried, dissolved in 0.2 ml of chloroform, and applied to a 0.5 × 50-cm column of Adsorbosil 100–200 mesh (Waters and Co.) in the presence of Adams catalyst. The sample was dried, dissolved in 0.2 ml of chloroform, and applied to a 0.5 × 50-cm column of Adsorbosil 100–200 mesh (Waters and Co.) in the presence of Adams catalyst. The sample was dried, dissolved in 0.2 ml of chloroform, and applied to a 0.5 × 50-cm column of Adsorbosil 100–200 mesh (Waters and Co.) in the presence of Adams catalyst.

The [3H]acetylphosphinganine was eluted with 8 ml of methanol. The thin layer chromatography step was repeated. The final product, with the same mobility as authentic N-acetylphosphinganine prepared according to a published procedure (26), had a specific activity of 1.5 × 10⁷ dpm/μmol.

**Miscellaneous Procedures—**Yeast were transformed using LiOAc-treated cells (27). Protein concentration was determined by using the Bradford reagent with bovine serum albumin as the standard (Bio-Rad).

### RESULTS

**Isolation of Mutant Strains Defective in Sphingolipid Synthesis—**To enrich for strains defective in sphingolipid synthesis we relied on the observation that a cell’s density increases when sphingolipid synthesis is blocked, presumably because the ratio of proteins to lipids increases (7). When SLC strains are fed PHS they make sphingolipids and have a normal density. If, however, a mutation has blocked sphingolipid synthesis the cell’s density should not increase, and the strain will not be made in the presence of PHS. This strategy allowed enrichment for SLC cells having a higher density following growth in the presence of PHS.

Putative mutants were screened to differentiate those specifically defective in sphingolipid synthesis from those defective...
in the text.

Cated, the origin is at 2.5 cm, and the identity of peaks 1 to 5 is discussed located by using a BioScan Apparatus. The solvent front (upper panels) one sample was given 25 μM PHS and the other was not. After 90 min of incubation (lower 4 panels), lipids were extracted, deacylated, and chromatographed on a silica gel thin layer plate. Radioactivity was located by using a BioScan Apparatus. The solvent front (F) is indicated, the origin is at 2.5 cm, and the identity of peaks 1 to 5 is discussed in the text.

in other lipid biosynthetic pathways which might also affect cell density. The screen was based upon the observation that strain 7R6 cannot grow at low pH when it lacks sphingolipids (no PHS present in the medium) but can grow when allowed to make sphingolipids (PHS present in the medium, Ref. 20). Thus, about 100 mutants unable to grow on PYED plates at pH 4.1 either in the presence or absence of 25 μM PHS were identified.

Mutant strains were examined further for a defect specific to sphingolipid synthesis relative to glycerophospholipid synthesis by looking for decreased incorporation of [3H]inositol into inositol-containing sphingolipids relative to phosphatidylinositol (28). By this assay, two strains, AG27 and AG84, appeared to be specifically defective in sphingolipid synthesis.

Both mutants tended to die rapidly even when stored in 15% glycerol at 70 °C. To preserve the mutants, each was crossed to strain YPH2 and the resulting diploids were stored frozen. Eventually the original haploid AG27 strain could not be revived from a frozen stock, so haploid offspring having the same phenotype as AG27 were obtained by sporulating the diploid and analyzing random spores. The phenotypes of AG27 are: Ura+; indicating the presence of the leb1-Δ:URA3 allele; Leb+, indicating the presence of the SLC1–1 suppressor gene; growth inhibition at 37 °C on PYED plates containing 25 μM PHS, indicating a block in sphingolipid synthesis (20). One such offspring, AG27-61, was used for the remainder of this research except for the data shown in Fig. 2, which were obtained using the original AG27 strain. Identical data to that shown in Fig. 2 were obtained using strain AG27-61 (data not shown).

**Strain AG27 Is Defective in IPC Synthase Activity**—The specific defect in sphingolipid synthesis in strain AG27 was determined in three steps. First, an in vivo radiolabeling procedure was used to determine if AG27 cells could make inositol-containing sphingolipids. Cells were cultured overnight, without PHS to prevent sphingolipid synthesis, but with [3H]inositol to label PI, a substrate for synthesis of IPC and M(IP)2C. Cells were separated from unincorporated radioisotope and incubated with or without PHS in a buffered solution containing glucose. The parental strain 7R6, but not the presumptive mutant strain AG27, should exhibit a pyrophosphoglycerin-dependent transfer of the radiolabel from phosphatidylinositol to ceramide to yield [3H]IPC and to [3H]MIPC to yield [3H]M(IP)2C. Strain 7R6 showed the three expected sphingolipids (peaks 3, 4, and 5; M(IP)2C, MIPC, IPC, respectively) only when the cells were incubated with PHS (Fig. 2). In contrast, identical amounts of AG27 cells did not exhibit these peaks indicating a defect in transferring the phosphoinositol of phosphatidylinositol to sphingolipids. Both strains showed deacylation products near the origin (Fig. 2, peaks 1 and 2) derived from deacylation of phosphatidylinositol, indicating that synthesis of phosphatidylinositol was normal. These results indicate that the mutation in strain AG27 either blocks uptake of long chain base, or inactivates ceramide synthase or IPC synthase.

Second, sphingolipid synthesis in mutant strain AG27, parental strain 7R6, and wild type strain SJ21R was compared using a membrane-permeable ceramide, [3H]N-Acetylphosphoryl-N-acetylsphinganine, to radiolabel sphingolipids, specifically IPC. After 5 h incubation, strains SJ21R and 7R6, but not strain AG27-61, convert [3H]N-acetylsphinganine in vivo to putative inositol-phosphoryl-N-acetylsphinganine, a non-deacylatable product that chromatographs on a thin layer plate at about 10 cm, slightly slower than the internal standard, yeast IPC-3 (Fig. 3). One would not expect these IPCs to migrate identically since the radioactive product would have 24 fewer methylenes and 2 fewer hydroxyls than the yeast IPC-3. In addition to its stability under deacylation conditions, the putative inositol-phosphoryl-N-acetylsphinganine is susceptible to “phosphatidylinositol-specific” phospholipase C; treatment with this enzyme results in its disappearance and appearance of [3H]N-acetylphosphoryl-N-acetylsphinganine as shown by thin layer chromatography (data not shown).

![Fig. 2. Strain AG27 does not incorporate [3H]inositol into sphingolipids.](image-url)
The failure of AG27 cells to incorporate $[^3H]$N-acetylsphinganine into IPC is even more dramatic when the time course of incorporation is examined quantitatively (Fig. 4). Strain 7R6 converts $[^3H]$N-acetylsphinganine to the IPC product at a much faster rate than does the wild type strain probably because strain 7R6 has no endogenous ceramide to compete with the radiolabeled non-physiological ceramide, whereas, the wild type strain contains endogenous ceramide. These results indicate that AG27-61 cells lack IPC synthase activity.

Finally, IPC synthase activity was assayed in microsomal membranes using the improved assay procedure described under "Experimental Procedures." This assay measures incorporation of $[^3H]$N-acetylsphinganine into total sphingolipids and gives a sphingolipid profile similar to the in vivo radiolabeling procedure as measured by thin layer chromatography of the radiolabeled products (data not shown). Membranes from wild type strain SJ21R and its SLC derivative 7R6 gave enzyme activity (Fig. 5) of about 4 nmol of product/mg protein/h which is in the range expected from enzyme activity measurements made in vivo (5). In contrast, membranes from strain AG27-61 contained little if any enzyme activity, the slight increase at the 30-min time point being within background values found over the course of many assays. Based upon the data shown in Figs. 3–5 we conclude that strain AG27-61 is unable to make sphingolipids because it lacks or has very reduced IPC synthase activity.

Exogenous Phytosphingosine Kills AG27 Cells—Early in the characterization of strain AG27 we noted sensitivity to exogenous PHS, but it was not clear whether cells were growth-inhibited or killed. To decide which of these possibilities was correct, cells were incubated at a low cell density with PHS and colony forming ability, a measure of viability, was determined. After 5 h of treatment with 10 $\mu$M PHS the viability of AG27-61 cells dropped from 100% to about 0.3% (Fig. 6). At 24 h viability increased slightly to about 4% but there was no increase in culture density, indicating little or no growth. In the absence of PHS, AG27-61 cells grew after an initial lag and were 100% viable at all time points. Growth of the 7R6 control cells was not inhibited by PHS and, in fact, the cells grew faster and to a higher density in the presence of PHS, probably because they were able to make sphingolipids. Viability was not reduced by PHS at any time point. Thus 7R6 cells are not killed by exogenous PHS but AG27-61 cells are.

The lcb1 gene is deleted in AG27-61 cells and they are thus unable to make endogenous long chain bases. If AG27-61 cells are killed by long chain base then they should not tolerate the wild type LCB1 gene. This hypothesis was verified by comparing the ability of AG27-61 and 7R6 to be transformed with a centromeric vector carrying the LCB1 gene (pLCB1-5, URA3). Only strain 7R6 gave transformants (Ura$^+$) indicating that strain AG27-61 would not tolerate the LCB1 gene. The parental vector lacking the LCB1 gene (pRS316, URA3) was able to transform both strains to Ura$^+$. Finally, strain AG27-61 was
treated with a mixture of the two plasmids. Plasmid DNA from was determined as described under “Experimental Procedures.”

Plasmid DNA from four PHS-resistant colonies was rescued in E. coli. The four plasmids, termed pIPC1, gave the same EcoRI restriction fragment pattern, indicating that they carry the same genomic DNA region. Each of the four pIPC1 DNA samples gave transformants when retransformed into AGD27-61 and selected on defined medium lacking leucine and containing 1 or 12 μM PHS whereas AGD27-61 transformed with the vector pRS315 gave no PHS-resistant transformants at the higher PHS concentration. These data show that the PHS-resistance phenotype is carried on pIPC1.

The sphingolipid synthesis defect in AG27-61 cells, haploid or diploid, prevents them from growing on PYED plates having a pH of 4.1 (PYED-4.1) when PHS is present whereas the parental strain 7R6 is able to grow because it makes sphingolipids (20). If pIPC1 restores sphingolipid synthesis then AGD27-61 cells transformed with the plasmid should behave like 7R6 cells and grow on PYED-4.1 plates containing 25 μM PHS. This expectation was fulfilled by all 30 AGD27-61 transformants tested while cells transformed with the vector did not grow. These data indicate that pIPC1 carries a gene that complements the IPC synthase defect in AGD27-61 cells and restores enzyme activity.

To identify the genomic region carried in pIPC1, the nucleotide sequence at the ends of the genomic insert was determined and used to search the Saccharomyces genome database at Stanford University. This search identified a region of 4307 nucleotides in Chromosome XI between coordinates 432,813 and 437,119. There is only one complete open-reading frame (YKL004w, AUR1) in this interval.

Because AGD27–61 diploids do not sporulate it was not possible to show by genetic analysis that AUR1 is linked to the IPC synthase defect. Instead, complementation (measured as resistance to PHS and growth at low pH) using portions of AUR1 localized a mutation to the 3’ end of the gene. For example, a purified 946-base pair NsiI-PvuII DNA fragment, representing the 3’ end of AUR1, restored PHS resistance and growth at low pH when transformed into AGD27-61 cells, but a purified DNA fragment corresponding to the 5’ end of the gene did not complement. The aur1 gene was retrieved from strain AGD27-61 by gap repair (30) of pIPC1. The DNA sequence of two independent clones was determined between the unique NsiI site in the coding region and the stop codon. Both clones lacked the C found in codon 325 of the wild type allele. The deletion allele is predicted to encode a variant protein of only 367 amino acids instead of 401 (see “Discussion”).

The AUR1 Gene Restores IPC Synthase Activity to AG27–61 Cells—Membranes from AG27–61 cells transformed with pIPC1 contained about 50% more IPC synthase activity than did membranes from the 7R6 positive control cells (Table I) while membranes from AGD27–61 cells contained barely detectable enzyme activity (Table I). Restoration of IPC synthase activity by pIPC1 was also examined by measuring incorporation of [3H]-acetylphosphine into cells by sphingolipids, specifically IPC. The concentration of radiolabeled IPC increased in A27–61 cells transformed with pIPC1 in an almost
linear fashion over the 3-h course of the experiment and, thus, these cells behaved like the positive control cells containing IPC synthase activity (7R6 transformed with pRS315) whereas AG27-61 cells transformed with pRS315 showed no synthesis of radiolabeled product (Fig. 8). Based upon the data presented in Table I and Fig. 8 we conclude that pIPC1 carries a gene capable of restoring IPC synthase activity in AG27-61 cells.

**IPC Synthase Activity Is Inhibited by Aureobasidin A**—Mutations in the *AUR1* gene have been shown to make *S. cerevisiae* cells resistant to AbA (14, 15). An important prediction of these results and our results is that AbA should inhibit IPC synthase activity. The data shown in Fig. 9 demonstrate that AbA strongly inhibits IPC synthase activity, with 50% inhibition (IC50) occurring at about 0.2 μM AbA.

**DISCUSSION**

Using the experimental rationale outlined under Introduction we have isolated a mutant strain defective in IPC synthase activity, the first time such a mutant has been isolated in any organism. A defect in IPC synthase activity was verified by demonstrating in vivo that mutant cells are unable to transfer [3H]inositol from [3H]PI to ceramide to make [3H]IPC (Fig. 2) and are unable to incorporate [3H]N-acetylphosphoglycerine into IPC (Figs. 3 and 5) whereas parental 7R6 and wild type SJ21R cells can incorporate both radiolabels into IPC. Direct assay of IPC synthase activity showed that membranes from AG27-61 cells transformed with pRS315 showed no synthesis of radiolabeled product (Fig. 7). Based upon the data presented in Table I and Fig. 8 we conclude that pIPC1 carries a gene capable of restoring IPC synthase activity in AG27-61 cells.

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**DISCUSSION**

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Ceramide, produced intracellularly from sphingomyelin, or exogenously added C_{26}-ceramide, induces programmed cell death (apoptosis) in a variety of animal cells by an incompletely characterized signal transduction pathway that includes a ceramide-activated protein kinase (36–38). It is not known if \textit{S. cerevisiae} has a ceramide-activated protein kinase nor if it has a process analogous to apoptosis, but based upon the ability of exogenous PHS to produce a large increase in the intracellular concentration of ceramide and kill AG27 cells or the intolerance of AG27-61 cells for a functional \textit{LCB1} gene, it seems possible that \textit{S. cerevisiae} has a ceramide-activated death response.

Ceramide accumulation in AG27 cells may prevent synthesis of suppressor lipids which are thought to compete with ceramide synthesis for \textit{C}_{26} fatty acid (13). Thus blockage of both sphingolipid and suppressor lipid synthesis, in addition to the ceramide-activated death pathway(s), could contribute to cell death.

Aureobasidin A inhibits growth of a wide range of fungi including the human pathogens \textit{C. albicans}, \textit{Cryptococcus neoformans}, \textit{H. capsulatum}, and \textit{Blastomyces dermatitidis}, and is fungicidal against \textit{Candida} (39) and \textit{S. cerevisiae} (15). Previous data from our laboratory explain why inhibition of IPC synthase is fungicidal. When sphingolipid synthesis is blocked, either by withdrawal of a long chain base from an auxotrophic strain (7) or by drug inhibition of the first enzyme in the pathway (serine palmitoyltransferase) (28), \textit{S. cerevisiae} cells die rapidly. These observations imply that any inhibitor of IPC synthase should be a broad spectrum fungicide. Now that \textit{AUR1} has been shown to be necessary for IPC synthase activity, it should be possible to develop high throughput screens to identify new IPC synthase inhibitors which are efficacious antifungal drugs.

REFERENCES


Fig. 10. Comparison of AUR1 proteins. The amino acid sequence of the predicted \textit{S. cerevisiae} AUR1 protein (ScAUR1) and a \textit{S. pombe} homolog (Z69086, SpAUR1) were aligned (40) to show amino acid identities (i) and similarities (s). Putative membrane-spanning domains are indicated by double underlines. The \textit{aour1} allele in strain AG27-61 (ScAUR1-27) has the C in codon 325 deleted. The effect of the resulting reading frameshift on the C terminus of the predicted variant protein sequence is shown in \textit{italics} above the wild type AUR1 protein sequence.

Phosphoalcohol group to a lipid alcohol acceptor forming a phosphodiester, suggest that AUR1p is a new member of a family of phospho-X transferases.

Our strategy for isolating mutants defective in the later steps in sphingolipid synthesis, starting with an SLC strain that can grow without making sphingolipids and cannot accumulate toxic sphingolipid intermediates, appears to have been justified based upon the findings that AG27-61 cells accumulate ceramide (Fig. 7) and are killed (Fig. 6) when fed exogenously added C_{2}-ceramide, induces programmed cell death. We verified this prediction by showing that AG27-61 could not be transformed with a centromeric vector carrying \textit{LCB1} (pLCB1-5) but could be transformed with the same vector lacking \textit{LCB1} (pRS316).

Comparison of our enrichment and screening procedure with the PHS-induced cell killing experiment presented in Fig. 6 presents an apparent conundrum: how did AG27 cells survive the enrichment and screening procedure since the agar plates contained 25 \textmu M PHS? We have observed that a higher concentration of PHS is needed to kill cells in solid than in liquid medium, so we think some mutants like AG27 survived because the concentration of PHS was too low to kill all of them. Had we used a lower concentration of PHS, more mutants like AG27 probably would have been recovered.
Sphingolipid Synthesis as a Target for Antifungal Drugs: COMPLEMENTATION OF THE INOSITOL PHOSPHORYLCERAMIDE SYNTHASE DEFECT IN A MUTANT STRAIN OF SACCHAROMYCES CEREVISIAE BY THE AUR1 GENE
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