Synthesis of Mannose-(inositol-P)$_2$-ceramide, the Major Sphingolipid in Saccharomyces cerevisiae, Requires the IPT1 (YDR072c) Gene*

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Knowledge of the Saccharomyces cerevisiae genes and proteins necessary for sphingolipid biosynthesis is far from complete. Such information should expedite studies of pathway regulation and sphingolipid functions. Using the Aur1 protein sequence, recently identified as necessary for synthesis of the sphingolipid inositol-P-ceramide (IPC), we show that a homolog (open reading frame YDR072c), termed Ipt1 (inositolphosphotransferase 1) is necessary for synthesis of mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C), the most abundant and complex sphingolipid in S. cerevisiae. This conclusion is based upon analysis of an ipt1-deletion strain, which fails to accumulate M(IP)$_2$C and instead accumulates increased amounts of the precursor mannose-inositol-P-ceramide. The mutant also fails to incorporate radioactive precursors into M(IP)$_2$C, and membranes prepared from it do not incorporate $[3^H$-inositol]phosphatidylinositol into M(IP)$_2$C, indicating a lack of M(IP)$_2$C synthase activity (putatively phosphatidylinositolmannose-inositol-P-ceramide phosphoinositol transferase). M(IP)$_2$C synthase activity is inhibited in the micromolar range by auroporphobilin A, but drug sensitivity is over 1000-fold lower than for IPC synthase activity. An ipt1-deletion mutant has no severe phenotypic effects but is slightly more resistant to growth inhibition by calcium ions. Identification of the IPT1 gene should be helpful in determining the function of the M(IP)$_2$C sphingolipid and in determining the catalytic mechanism of IPC and M(IP)$_2$C synthases.

Sphingolipids along with cholesterol and phosphoglycerolipids are the three major types of lipids found in biological membranes. The sphingolipids in Saccharomyces cerevisiae are primarily located in the plasma membrane (1, 2), where they account for 7–8% of the total mass of the membrane (30% of the plasma membrane phospholipids; Ref. 1). At present, we have only a general outline of the steps in S. cerevisiae sphingolipid biosynthesis (Fig. 1) and know nothing about how the concentration of the intermediates and complex sphingolipids is regulated. Further understanding of the pathway requires identification of the biosynthetic genes and purification and characterization of the cognate enzymes, none of which have been purified. Only three S. cerevisiae genes necessary for sphingolipid synthesis, LCBI (3), LCB2 (3–5), and AUR1 (6), have been identified. Completion of the S. cerevisiae genome sequence (7) should facilitate identification of the remaining genes. By using the S. cerevisiae genome sequence data base, we have now identified another sphingolipid biosynthetic gene, YDR072c, and renamed IPT1 (inositolphosphotransferase 1). This gene is necessary for synthesis of mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C; Ref. 8), the terminal and most abundant S. cerevisiae sphingolipid (Fig. 1).

Sphingolipid synthesis in S. cerevisiae begins with the condensation of serine and palmitoyl-CoA to yield 3-ketosphinganine. This essentially irreversible reaction is catalyzed by serine palmitoyltransferase (3-ketosphinganine synthase (EC 2.3.1.50); for review, see Ref. 9), a pyridoxal phosphate-containing enzyme. Further reactions convert 3-ketosphinganine to the long chain base sphinganine (dihydrosphingosine), which is N-fatty-acetylated to yield dihydroceramide. Dehydrogenation of dihydroceramide in animals yields ceramide with the long chain base sphingosine, which is rapidly converted to sphingolipids by the addition of polar components to the 1-hydroxyl group. Most ceramides in fungi and plants contain N-$\alpha$-hydroxy fattyacylphytosphingosine (8), formed by undefined hydroxylation reactions. Phytosphingosine lacks the 4,5-double bond found in sphingosine and has instead an hydroxyl group at the 4-position. Molecular species with the same head groups have been recognized (8) that differ in hydroxylations of the long chain base and the fatty acid. The most abundant species, designated 3, e.g. M(IP)$_2$C-3, contains phytosphingosine and aOH-hexacosanoic acid.

A common modification to phytoceramide in S. cerevisiae and other fungi is addition of myoinositol phosphate to the 1-hydroxyl to form IPC, which is then mannosylated to yield mannosyl-inositol-P-ceramide (MIPC). The terminal step in S. cerevisiae sphingolipid synthesis is addition of inositol-P to MIPC to yield the major sphingolipid M(IP)$_2$C. The later steps in sphingolipid synthesis in S. cerevisiae (Fig. 1) are tentative because the enzymes have not been purified, the reaction requirements are poorly defined, and the stoichiometry has not been determined (reviewed in Ref. 8).

We recently identified a gene, AUR1, necessary for phosphatidylinositol:ceramide phosphoinositol transferase (IPC synthase) activity (6). The gene most likely encodes the IPC synthase enzyme or a subunit of the enzyme whose subunit structure is unknown. IPC synthase appears to catalyze a reaction very similar to the one catalyzed by phosphatidylinositol:mannose-inositol-P-ceramide phosphoinositol transferase (M(IP)$_2$C synthase), since both transfer inositol-P from phosphatidylinositol to ceramide or a ceramide-containing compound. This similarity suggested that there might be a protein

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1 The abbreviations used are: M(IP)$_2$C, mannose-(inositol-P)$_2$-ceramide; IPC, inositol-phosphorylceramide; MIPC, mannose-inositol-P-ceramide; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
in *S. cerevisiae* with similarity to the Aur1 protein. Using the Aur1 protein as a query, we found a protein in the *S. cerevisiae* genome data base, encoded by *IPT1*, that appeared to be related to the Aur1 protein. We show here that *IPT1* is indeed necessary for M(IP)2C synthase activity and for synthesis of M(IP)2C, which was derived from strain YPH250 (10) by gene transplacement of the *IPT1* gene using the FASTA algorithm (18).

**Results**

Identification of an Aur1 Protein Homolog—Structural homologs of the Aur1 protein were searched for in the *S. cerevisiae* genome data base for nucleic acid sequences that share identity to the Aur1 protein over a region of 365 amino acids to the predicted Ipt1 protein, which shows 27% amino acid identity to the Aur1 protein (11). Correct gene transplacement was verified by Southern blot analysis (data not shown).

**Experimental Procedures**

**Strains, Media, and Culture Conditions—**Strain RCD113 (MATa ura3–52 leu2–016 ade2–101 his3–200 trp1–Δ1 ipt1–Δ1) was derived from strain YPH250 (10) by gene transplacement of the YDR072c (IPT1) open reading frame with the ipt1–Δ1 allele, made by replacing the coding region of *IPT1* with the *HIS3* gene using the polymerase chain reaction as described by Baudin et al. (11). Correct gene transplacement was verified by Southern blot analysis (data not shown).

**Analysis of Sphingolipids—**Cells were cultured at 21 h (initial *A_{600} = 0.1*) at 30 °C in a medium consisting of 1% peptone, 0.2% yeast extract, 0.05% Tergitol, 1% KH2PO4, 0.05 M sodium succinate, pH 5.5, 1 μg/ml inositol, 4% glucose, and 20 μCi/ml [2-14C]inositol (American Radiochemicals, Inc., 20 Ci/mmol). Cells (5 ml) were treated with trichloroacetic acid to a final concentration of 5% and chilled for 15 min on ice. After twice washing with water, cells were extracted with 2 ml of Solvent E, diethylether/95% ethanol/water/pyridine/concentrated acetic acid to a final concentration of 5% and chilled for 15 min on ice. After twice washing with water, cells were extracted with 2 ml of Solvent E, diethylether/95% ethanol/water/pyridine/concentrated acetic acid to a final concentration of 5% and chilled for 15 min on ice.

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**Identification of an Aur1 Protein Homolog—**Structural homologs of the Aur1 protein were searched for in the *S. cerevisiae* genome data base for nucleic acid sequences that share identity to the Aur1 protein over a region of 365 amino acids (11). Only one putative homolog, Ipt1 (YDR072c, GenBank accession no. Z46796x5), was identified. No function had been attributed to the predicted Ipt1 protein, which shows 27% amino acid identity to the Aur1 protein over a region of 365 amino acids (Fig. 2).

**To determine if **IPT1** is necessary for synthesis of **M(IP)2C**, sphingolipids were radiolabeled by growing cells long term in the presence of [3H]myoinositol. Extracted radioactive sphingolipids were either subjected to or not subjected to alkaline-catalyzed deacylation, separated by high performance TLC, and located by using a BioScan apparatus. Comparison of the scans (Fig. 3) to the authentic S. cerevisiae sphingolipid internal standards shows that the ipt1 deletion strain RCD113 lacks the radioactive fraction representing the alkali-stable M(IP)2C species present in the scans of the lipids isolated from wild type YPH250 cells. Mutant strain RCD113 shows an increase in radiolabeled MIPC-3 (Fig. 3), indicating that this
species of sphingolipid accumulates.

Mutant cells carrying the SLC1–1 suppressor gene and unable to make sphingolipids when a long chain base is withheld from the culture medium, accumulate inositol-containing lipids in which the ceramide is replaced with diacylglycerols containing a C26-fatty acid (19, 20). No such lipids are evident in RCD113 cells (Fig. 3, bottom).

To verify the absence of M(IP)2C and an increase in MIPC-3 in strain RCD113, non-radioactive deacylated lipid extracts were again analyzed by TLC and the plate was treated with the orcinol reagent to detect carbohydrates (mannose) and then charred to detect all the carbon-containing polar head groups. This analysis shows that M(IP)2C is not present in strain RCD113 and that the concentration of MIPC is elevated (Fig. 4). The identity of these lipids was confirmed by their reaction with the orcinol reagent.

Strain RCD113 Lacks M(IP)2C Synthase Activity—M(IP)2C synthase activity was measured in membranes prepared from YPH250 and ipt1-mutant RCD113 cells by the incorporation of radiolabeled inositol into M(IP)2C from [3H-inositol]phosphatidylinositol. The reaction catalyzed by M(IP)2C synthase is not well characterized (8) but is believed to use MIPC and phosphatidylinositol as substrates. The [3H]M(IP)2C produced in the reaction was first analyzed by thin layer chromatography to verify that the correct product was being made. Membranes prepared from YPH250 but not from the mutant RCD113 cells make [3H]M(IP)2C, as judged by its co-migration with the internal M(IP)2C standard (Fig. 5). Reactions conditions were then examined in more detail. Membranes from YPH250 cells produced [3H]M(IP)2C linearly with time and with the protein concentrations examined. No production of [3H]M(IP)2C was observed with membranes from RCD113 cells (Fig. 6). Omission of exogenous MIPC from the reaction mixture (Table I) reduces product formation by about 65%, consistent with this lipid being a substrate for the enzyme. We conclude from these data that RCD113 cells lack M(IP)2C synthase activity.

M(IP)2C Synthase Activity Is Inhibited by Aureobasidin A—IPC synthase activity, presumably encoded by AUR1, is strongly inhibited by the antifungal drug aureobasidin A (6). Since the Ipt1 protein is related to the Aur1 protein and each is required for expression of the IPC and M(IP)2C synthases, respectively, and since both enzymes utilize phosphatidylinositol as one of their substrates, catalyzing a similar phosphoinositol transfer, it was logical to examine the inhibition of M(IP)2C synthase by aureobasidin A. The data presented in Table I show that aureobasidin A strongly inhibits M(IP)2C synthase in the micromolar range, with 50% inhibition (IC50) at 0.5–1 μM.

Phenotypes of Strain RCD113—To begin to understand the function of M(IP)2C, we compared the behavior of RCD113 mutant and wild type YPH250 cells grown under a variety of conditions. Examination of mutant and wild type cells in log
and stationary phase growth by light microscopy showed no difference in their size or morphology. Growth in a complex medium at 30 °C showed that RCD113 mutant cells grew as rapidly as parental YPH250 cells and to the same density during the course of this experiment (Fig. 7), indicating that the lack of M(IP)2C had no adverse effect upon growth rate during log phase and early stationary phase growth nor upon the ability of cells to grow to a fairly high density.

We had shown previously (21) that one or more sphingolipids are necessary for

Saccharomyces cerevisiae cells to grow under stress conditions including incubation at 37 °C, containing 0.75 M NaCl and at low pH. To determine if M(IP)2C is necessary for growth under these stress conditions, RCD113 mutant cells were tested for growth on PYED complex medium plates incubated at 37 °C, containing 0.75 M NaCl and incubated at 30 °C, or having a pH of 4.1 and incubated at 30 °C (12). RCD113 cells grew as well as wild type cells under all conditions (data not shown). Thus, M(IP)2C is not necessary for responding to these stresses.

We have also observed that mutant yeast cells lacking sphingolipids (21) are unable to be induced for what has been termed induced thermotolerance, in which survival at an elevated temperature (greater than about 45 °C) can be enhanced by preincubation at an intermediate temperature, generally 37 °C (reviewed in Ref. 22). To determine if M(IP)2C lipids play a role in induced thermotolerance, early log phase RCD113 and YPH250 cells were incubated at 25 °C or 37 °C for 30 min and transferred to 52 °C, and surviving cells were measured at 0, 20, and 40 min. Survival was the same for both strains, and both survived the 52 °C treatment better after preincubation at 37 °C, indicating that thermotolerance had been induced (data not shown). We conclude from these data that induction of thermotolerance does not require M(IP)2C lipids.

Sphingolipids are believed to reach the plasma membrane by the protein secretory pathway. Most sphingolipids would ini-
Saccharomyces Mutant Lacking the Major Sphingolipid

Here, we present evidence that the IPT1 gene is necessary for synthesis of M(IP)_2C, the major and terminal sphingolipid in *S. cerevisiae*. This assignment is based upon that fact that ipt1-deleted RCD113 cells fail to make M(IP)_2C and, instead, make increased amounts of MIPC (Figs. 3 and 4), the precursor to M(IP)_2C (Fig. 1). In addition, membranes prepared from RCD113 cells lack M(IP)_2C synthase activity since they fail to make [³H]M(IP)_2C from [³H-inositol]phosphatidylinositol (Figs. 4 and 5).

The observation that mutant RCD113 cells compensate for the loss of M(IP)_2C lipids and make a nearly equal amount of MIPC lipids indicates that *S. cerevisiae* cells have a mechanism for sensing their total sphingolipid content. MIPC and M(IP)_2C may be alternate fates of the ceramide available for sphingolipid synthesis.

Attempts to identify a function for M(IP)_2C lipids were inconclusive since RCD113 mutant cells grew as well as non-mutant cells in both complex and defined media and under a variety of stress conditions that are known to inhibit growth of cells that lack sphingolipids (21). RCD113 cells may not exhibit obvious phenotypes because the elevated level of MIPC may compensate for some functions of M(IP)_2C.

The limited available data indicate that, as long as *S. cerevisiae* cells make at least one type of sphingolipid, they are viable under non-stressful laboratory conditions. For example, mutant cells lacking mannosylated sphingolipids (5), but containing at least one species of IPC, grow well if given 10 mM calcium. However, these mutants have not been extensively tested for mutant phenotypes. *S. cerevisiae* cells lacking any sphingolipids grow under non-stressful conditions but are killed by stresses, indicating a role(s) for sphingolipids in stress resistance (21). Finally, no wild type fungus has been reported to exist without making sphingolipids. In addition to *S. cerevisiae* (26), *Cryptococcus neoformans* (27) and *Candida albicans* (28) are known to contain M(IP)_2C, whereas *Neurospora crassa* contains a major lipid with the composition (inositol-P_2-ceramide (28). *Histoplasma capsulatum* makes several mannosylated sphingolipids but not M(IP)_2C when growing in the yeast phase (29, 30).

RCD113 cells were slightly more resistant to the growth inhibitory effect of calcium ions than were non-mutant YPH250 cells (Table II). Mutations in seven complementation groups, SCS1–7, give resistance to calcium-inhibited growth of a strain mutated in the csg2 gene (5). The scs mutant strains all have altered sphingolipid metabolism, and one complementation group, SCS1, is identical to the LCB2 gene (4), necessary for the first step in sphingolipid synthesis (Fig. 1). Based upon these results, it has been suggested that sphingolipid metabolism in *S. cerevisiae* is either regulated by Ca^{2+} and/or is required for Ca^{2+} homeostasis (5). The calcium resistance phenotype of RCD113 cells is consistent with these possibilities.

Finally, because their synthesis has been conserved and because of their abundance, the M(IP)_2C lipids must perform an important function(s) that awaits identification.

M(IP)_2C synthase activity is found in membranes (33). Analysis of the Ipt1 protein sequence indicates seven predicted membrane-spanning domains (34) (Fig. 2). Several of these transmembrane domains correspond to predicted transmembrane domains in the Aur1 protein (Fig. 2), suggesting that these proteins have related membrane topologies. However, the predicted inside-outside orientation of some of the transmembrane domains are opposite in the two proteins, and experimental analysis will be required to verify the correct topology and existence of the transmembrane domains.

IPC synthase activity is strongly inhibited by the antifungal drug aurobasidin A, with 50% inhibition seen at a concentration of 0.2 nm (6). Because M(IP)_2C synthase and IPC synthase

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**Fig. 7. Growth rate of mutant and wild type cells.** Parental YPH250 (●) and ipt1-deleted RCD113 (○) cells were grown in PYED complex medium in shaker flasks at 30 °C. The cell density was measured at 600 nm (A_{600}) by using a spectrophotometer. Most data points are superimposed.

| Calcium | A_{600 nm} | YPH250 | RCD113 (ipt1-ΔI) |
|---------|------------|--------|------------------|
| 0       | 1.66       | 2.1    |                  |
| 10      | 1.87       | 2.3    |                  |
| 50      | 0.32       | 1.22   |                  |
| 100     | 0.096      | 0.31   |                  |
| 200     | 0.023      | 0.083  |                  |

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**DISCUSSION**

Here, we present evidence that the IPT1 gene is necessary for synthesis of M(IP)_2C, the major and terminal sphingolipid...
both use phosphatidylinositol as a substrate (Fig. 1) and because of their amino acid sequence similarity (Fig. 2), we examined M(IP)2C synthase for inhibition by aureobasidin A. Our data demonstrate that M(IP)2C synthase activity is strongly inhibited by the drug (Table I), but the enzyme is 2500–5000-fold less sensitive to inhibition than is IPC synthase activity. Further kinetic experiments with both enzymes, native and mutant forms (see below), along with analogues of aureobasidin, could be fruitful in pinpointing the catalytic sites in these enzymes.

Mutations in the AUR1 gene are known to give resistance to aureobasidin A. One resistant strain has Leu-137 replaced by Phe and His-157 replaced by Tyr (35), whereas another resistant strain has Phe-158 replaced by Tyr (36). Residues 157 and 158 are particularly interesting because they lie in a region whose amino acid sequence has been well conserved between the Aur1 and Ipt1 proteins (Fig. 2). This region is predicted to be a membrane-spanning domain and since there would be no strong evolutionary pressure to conserve the amino acid sequence simply to maintain the membrane-spanning function, the conservation of residues suggests some other shared function. We speculate, based upon the amino acid conservation, the predicted transmembrane domains, and the location of the aureobasidin A-resistant mutations, that the region of Aur1p from around residue 146 (residue 186 in Ipt1p) to around 259 (residue 302 in Ipt1p) is the catalytic core of the two enzymes that interacts with the hydrophobic portion of one or both lipid substrates. Furthermore, we speculate that the conserved nonmembrane-bound region from around residue 300 in Aur1p (residue 323 in Ipt1p) to the C terminus of the protein (residue 409 in Ipt1p) recognizes the polar head-group in phosphatidylinositol. The corresponding regions in Ipt1p would perform similar functions.

The availability of the complete S. cerevisiae genomic sequence provides the first opportunity to identify all genes and proteins necessary for sphingolipid synthesis in any organism. Knowledge of the genes should enable characterization of the cognate proteins and enzymes and this information should expedite the task of understanding how sphingolipid synthesis is regulated in S. cerevisiae. Since it is not known how sphingolipid synthesis is regulated in any organism, insight gained from S. cerevisiae may provide clues to regulation in other eucaryotes. In addition, the level of sphingolipid metabolic intermediates, dihydrosphingosine, phytosphingosine and ceramide, thought to be signaling molecules that regulate expression of at least some of the many yeast genes whose promoter contains an STRE (stress response element), increases during heat stress by an unknown mechanism. Knowledge of sphingolipid biosynthetic genes and proteins should facilitate studies to understand how heat stress regulates the level of these intermediates.

Addendum—After submission of this work for publication, Leber et al. (31) reported on a mutant strain that does not make M(IP)2C when grown at the restrictive temperature. It is not known whether their strain is mutated in the IPT1 or some unknown gene that affects M(IP)2C synthesis. Their mutant strain, like ours, has no growth defects under a variety of conditions. They did observe that the mutant strain was more resistant than the wild type strain to the polyene antibiotic nystatin. Nystatin is thought to cause cell death by interacting with sterols and forming pores in the plasma membrane (32). They suggest that M(IP)2C, in addition to ergosterol, is necessary for nystatin action on membranes and that the two types of lipids may exist as microdomains within the plasma membrane. It needs to be determined whether M(IP)2C lipids have a propensity to interact with ergosterol and whether they do so better than MIPC and IPC.

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