Cloning of an Alkaline Ceramidase from Saccharomyces cerevisiae

AN ENZYME WITH REVERSE (CoA-INDEPENDENT) CERAMIDE SYNTHASE ACTIVITY*¹

(Received for publication, November 2, 1999, and in revised form, December 4, 1999)

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Ceramide synthase has been purified or cloned. Ceramide synthase activity has been detected in mammalian cells (9). Ceramidase activity is also a target of a fungal toxin, fumonisin B1. Here we describe this approach, and we provide evidence explaining why YPC1 was cloned as a fumonisin B1-resistant gene.

Ceramide synthase activity has been detected in mammalian cells (5) and yeast cells (6). It catalyzes the acylation of sphingosine from palmitic acid and phytosphingosine. This ceramide synthase activity is CoA-independent and is resistant to fumonisin B1, thus explaining why YPC1 was cloned as a fumonisin B1-resistant gene.

Ceramide forms the backbone of complex sphingolipids in mammalian cells. Ceramide, its breakdown product sphingosine, and its metabolite sphingosine-1-P are important signaling molecules that mediate different cellular events including apoptosis, growth arrest, stress responses, and proliferation (see reviews Refs. 1–4). As a signaling molecule, turnover of ceramide must be tightly regulated. Ceramide synthase and ceramidase catalyze synthesis and degradation of ceramide, respectively.

Ceramide synthase activity has been detected in mammalian (5) and yeast cells (6). It catalyzes the acylation of sphingosine by a fatty acyl-CoA. Additional studies show that ceramide can also be synthesized in vitro from sphingosine and palmitic acid in a CoA-independent reverse activity of a sphingolipid ceramide deacylase (7) or an alkaline ceramidase (8). To date no ceramide synthase has been purified or cloned.

Several ceramide activities responsible for the breakdown of ceramide have been detected in mammalian cells (9). Ceramidases are classified as acidic, neutral, or alkaline based on their pH optimum. Acidic ceramidase is localized in lysosomes and is responsible primarily for catabolism of ceramide (10). This enzyme has been identified, and its cDNA has been cloned from human (11) and mouse (12). On the other hand neutral and alkaline ceramidases have been implicated in signal transduction and cell regulation (13, 14). One of these enzymes has recently been characterized and purified to homogeneity (15).

In mammals, dihydroceramide and phytoceramide are important intermediates of complex sphingolipids in the yeast Saccharomyces cerevisiae (4). Several studies have implicated dihydrophytoceramide, dihydrophytosphingosine, and dihydrophytosphingosine 1-phosphate in mediating regulation of cell growth and stress responses in yeast (16–18). Consequently, cellular levels of these sphingolipids have to be regulated either by their immediate conversion to more complex sphingolipids or by their breakdown. Coordinated action of multiple metabolic enzymes is a key to fulfill this task of regulation and underscores the importance of this regulation for signaling processes in S. cerevisiae. Identification of these enzymes will facilitate our understanding of the signaling processes mediated by sphingolipids.

We elected to apply yeast genetic techniques to clone ceramide synthase. This enzyme is also a target of a fungal toxin, namely fumonisin B1, which inhibits cell growth by suppressing synthesis of sphingolipids (6). We therefore used the strategy of overexpressing a high copy yeast genomic DNA library to identify genes whose overexpression endows resistance to growth suppression by fumonisin B1. By using this strategy we identified a gene whose overexpression imparts on cells the ability to synthesize sphingolipids in the presence of fumonisin B1. Here we describe this approach, and we provide evidence that the gene we obtained, YPC1, is in fact encodes a yeast alkaline ceramidase. Moreover, YPC1p also has the reverse activity of catalyzing ceramide formation from phytosphingosine and palmitic acid independent of coenzyme A, thus explaining why we obtained this enzyme as a fumonisin B1-resistant gene.

MATERIALS AND METHODS

Yeasts and Bacterial Strains—Yeast strains used in this study are listed in Table I. The mutant strain Δyor1 was derived from the wild type JK9–3d α by replacing a portion (from the start codon to nt 1073) of the YOR1 coding sequence with the G418-resistant gene (KanMX) as

1 The abbreviations used are: YPC1, the Yeast Phytoceramidase; YPC1p, the product of the YPC1 gene; G418, geneticin; nt, nucleotide; PCR, DNA polymerase chain reaction; ORF, open reading frame; DHS, dihydrosphingosine; DHS-1-P, dihydrosphingosine 1-phosphate; PHS, phytosphingosine; PHS-1-P, phytosphingosine 1-phosphate; IPC, inositol phosphoceramide; MIPC, mannosylated IPC; ER, endoplasmic reticulum.
described (19). Wild type and mutant strains were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose, Difco). Plasmid-encoding strains were grown in a uracil dropout medium SC-ura medium (1% yeast extract, 2% peptone, and 2% dextrose, Difico). Plasmids were isolated from these 20 transformants by electroporation. Plasmids were amplified and purified as described (20).

**Construction of the Yeast Genomic DNA Library—**DNA was isolated from the yeast strain Δyor1 as described (20). To remove RNA, RNase (free from DNase I) was added to DNA preparations and incubated at 37°C for 30 min. DNA was partially digested with the diluted Sau3A1 for 20 min. Sau3A1-digested DNA was run on 0.8% agarose gel, and 4–6-kilobase DNA fragments were excised and purified by a Ultra-Free-DA unit (Amicon) as suggested by the manufacturer. After being extracted with phenol:chloroform (25:80) followed by ethanol precipitation, the DNA fragments were dissolved in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA.

**Preparation of Radiolabeled Ceramides—**[3H]Ceramide and phytoceramide were synthesized as follows: N-[9,10-3H]erythro-C16-ceramide and N-[9,10-3H]ribo-C16-phytoceramide were prepared by acylation of the respective sphingoid bases with [9,10-3H]palmitoyl chloride generated in situ from [9,10-3H]palmitic acid as described (23). E-erythrospingosine was obtained in stereo- and enantio-specific synthesis from hydrolysis of the mixed enantiomer of [9,10-3H]C-6-dihydrosphingosine (C6-D-erythro-[4,5-3H]) by dihydroinosphingosine, and E-erythro-[4,5-3H]dihydroinosphingosine 1-phosphate were from American Radiolabeled Chemicals (ARC, Inc.).

**Preparation of Microsomes—**Microsomes were isolated from the yeast cells as described (20) with minor modification as follows. Cells were suspended in a lysis buffer (25 mM Tris-HCl, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride and 20 µg/ml CLAP) after being washed twice with ice-cold water. Cells were sonicated and centrifuged at 4,000 rpm for 5 min. Untouched cells were centrifuged again for 5 min. Unbroken cells and cell debris were removed by centrifugation at 4,000 rpm for 5 min. To pellet the membrane fraction, the supernatant was centrifuged at 40,000 rpm for 40 min at 4°C. The membrane fraction was rinsed gently with the lysis buffer and suspended in an appropriate assay buffer. Protein concentrations were determined using a Bradford reagent (Bio-Rad).
were dried on a SpeedVac and dissolved in 20 mM serially diluted. Three µl of cells from each dilution were spotted onto SC-ura medium with or without fumonisin B1 and incubated at 30°C for 3 days. The plates were photographed on a ChemImage system (Alpha-Inotech). A, cells with an empty vector (YEpplac195) or with the vector containing inserts (clones 1–3) were grown on SC-ura plates containing 2% glucose; B, cells of the strain Δyor1-Vec or Δyor1-YPC1 were grown on SC-ura plates containing 2% galactose. Fum, fumonisin B1.

150–300 µg of proteins) of microsomes were added to substrates, and reactions were incubated at 30°C for 40–90 min. Both the protein concentration and the time of incubation were within the linear range for the assay. The reactions were terminated by adding 200 µl of chloroform:methanol (2:1, v/v). Reaction mixture was dried and dissolved in 50 µl of chloroform:methanol (2:1, v/v), 25 µl of the mixture was applied onto a silica gel 60A TLC plate (Whatman) and resolved by the solvent system I (chloroform, methanol, 25% ammonium hydroxide, 9:2:0.5, v/v) (25). Free palmitic acid or DHS, the reaction product separated from the substrate by TLC, was identified according to a standard and quantified by a scintillation counter. One unit of ceramidase was defined as the amount of the enzyme needed to release 1 pmol of palmitic acid or dihydrosphingosine per min.

To measure the activity of ceramide synthase independent of fatty acid-CoA, phytosphingosine (5 mmol) and [3H]palmitic acid (0.3 mmol) were dried on a SpeedVac and dissolved in 20 µl of the buffer A as described above. Twenty µl of microsomes were added to the above substrates and incubated at 30°C for 4–5 h. Both protein concentration and time of incubation were within the linear range for the assay. Reactions were terminated as described above. Ceramide formation was analyzed by the same TLC system with C16-phytoceramide as a standard. One unit of ceramidase synthase was defined as the amount of the enzyme needed to form 1 pmol of phytoceramide per min.

Sphingolipid Labeling—Cells (3 × 107 in 1 ml of medium) were labeled with [3H]palmitic acid, serine, or C-6-dihydroceramide (5–10 µCi) at 30°C for different periods. Total lipids were extracted, deacylated by monomethylamine (20% in ethanol), and resolved by TLC using the solvent system II (chloroform, methanol, 4.2 N ammonium hydroxide, 9:2:0.5, v/v) as described (26). TLC plates were sprayed with ENHANCE and radiographed on BioMax films (Eastman Kodak Co.). Radiolabeled sphingolipids were identified according to the authentic standards included on the same TLC plate. To quantify an individual lipid, the radioactive bands were scraped and counted by a scintillation counter (Beckman Instruments).

Protein Analysis—Proteins were separated by SDS-polyacrylamide gel electrophoresis and were detected by Western blotting analysis by following standard procedures.

RESULTS

Overexpression of YPC1 Imparts Resistance to Fumonisin B1—In order to clone the S. cerevisiae ceramide synthase, we set out to screen for genes whose overexpression imparts resistance to fumonisin B1, using a high copy yeast genomic DNA library. The yeast strain we used for this screen had a deletion in this strain more sensitive to fumonisin B1 and allowed us to use less fumonisin B1 for the screen. Upon screening this library we obtained 3 clones all of which were similar and encoded for a known yeast gene that is a member of the ATP-binding cassette (ABC) transporter family of genes namely YOR1 (yeast oligomycin resistance gene). The reason YOR1p endows resistance to fumonisin B1 appears to be due to its ability to pump out fumonisin B1 and/or other sphingolipids that may accumulate as a result of fumonisin B1 treatment. In order to pursue our initial goal to obtain ceramide synthase, we next elected to prepare a yeast genomic DNA library from the Δyor1 strain and to use this library to screen for genes that impart resistance to fumonisin B1 that are different from YOR1 as described under “Materials and Methods.” Of the 20 fumonisin B1-resistant transformants we analyzed, 16 were found to have plasmid-dependent resistance to fumonisin. Fig. 1A shows that cells transformed with three separate representative clones confer resistance to fumonisin B1. The inserts of the plasmids were sequenced from both ends, and a search of the Saccharomyces Genomic Data base with the partial sequences obtained derived the entire sequence of the inserts. Sequencing revealed that 7 out of 16 clones were identical, having the same insert, spanning three ORFs. The 9 other plasmids contained different inserts. However, all the clones shared a consensus sequence covering a hypothetical ORF YBR183w on chromosome II. YBR183w, designated as YPC1, was the only ORF in one of the plasmids. We speculated that YBR183w might confer resistance to fumonisin B1. To verify if that was the case, we amplified the ORF YBR183w from yeast genomic DNA by PCR and cloned it into the yeast expression vector pYES2 to create the plasmid pYES2-YPC1, thus ex-

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pressing YPC1p under the control of the Gal1 promoter. pYES2-YPC1 and the vector pYES2 were introduced into the strain Δyor1, respectively. The vector-containing strain (Δyor1-Vec) and the pYES2-YPC1-containing strain (Δyor1-YPC1) were grown in SC-ura medium with 2% galactose. The expression of the gene YPC1 was induced by galactose. Fig. 1B shows that the strain Δyor1-YPC1 but not Δyor1-Vec was resistant to fumonisin B1 in the galactose-containing medium. These data suggest that the resistance to fumonisin B1 is indeed attributed to overexpression of YPC1p.

Overexpression of YPC1p Changes the Metabolism of Sphingolipids—Examination of the YPC1p sequence predicted it to
have several transmembrane domains. It had high homology to another yeast hypothetical protein encoded by the ORF \textit{YPL087w}. A BLAST search showed that YPC1p did not have any significant homology to other proteins with known functions in all protein data bases searched, suggesting it is a novel protein. Motif search indicated that it contained an ATP-binding site (GXGXG(X\ldots X)_1pK, where $X$ indicates any amino acid residue) shared by human diacylglycerol kinase $\zeta$ (27) and many protein kinases. On its carboxyl terminus, it had an ER retention signal (KKXX), which is shared by several ER proteins including sphingolipid metabolic enzymes ketodihydrosphingosine reductase TSC10p (28) and dihydrosphingosine (or dihydroceramide) hydroxylase SYR2p (29), suggesting it might be localized to the ER.

We could not predict function of the product of the \textit{YPC1} gene based on protein homology. However \textit{YPC1} was selected for allowing resistance to fumonisin B1. Fumonisin B1 inhibited cell growth in the DHS-1-P lyase (DPL1) deletion because it led to accumulation of phosphorylated long chain bases and/or because it inhibited formation of ceramide and sphingolipids. Therefore, we speculated that overexpression of YPC1p could either lead to breakdown of accumulated long chain base phosphates or to bypassing the ceramide synthase block and generating ceramide and other ceramide-containing sphingolipids despite the presence of fumonisin B1. To investigate this, the strains \textit{\Deltayor1-Vec} and \textit{\Deltayor1-YPC1} were grown in SC-ura medium with galactose, which induced YPC1p expression. The cells were then treated with 100 $\mu$M fumonisin B1 for 2 h and labeled with palmitic acid for 4 h. Total lipids were extracted and resolved by TLC after base hydrolysis by monomethylamine as described under “Materials and Methods.” Autoradiography of the TLC plate (Fig. 2A) demonstrated that formation of the sphingolipids IPC, MIPC, and M(IP)2C was significantly blocked by fumonisin B1 in the vector control cells (\textit{\Deltayor1-Vec}) but not in the YPC1p-overexpressing cells (\textit{\Deltayor1-YPC1}). Quantitation showed that the \textit{\Deltayor1-YPC1} cells had 4 times more incorporation of palmitic acid into complex sphingolipids (IPC, MIPC, and M(IP)2C) than did the \textit{\Deltayor1-Vec} cells in the presence of fumonisin B1 (Fig. 2B). The data sug-

**Fig. 5.** \textbf{YPC1p has alkaline ceramidase activity.} The activity that hydrolyzed \textit{[1H]C-6-dihydroceramide} was measured as described in Fig. 4 with microsomes isolated from cells overexpressing YPC1p at different pH values. Sodium acetate buffer (25 mM) was used for pH 4.5–6, Tris-HCl buffer (25 mM) was used for pH 7–8, and glycine NaOH buffer (25 mM) was used for pH 9–10. All buffers contained 5 mM CaCl$_2$ and 0.25% Triton X-100. The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments.

**Fig. 6.** \textbf{YPC1 hydrolyzes phytoceramide but not unsaturated ceramide.} Microsomes were assayed for ceramidase activity from the \textit{\Deltayor1-Vec} and \textit{\Deltayor1-YPC1} cells toward phytoceramide (A) or ceramide (B) as described in Fig. 4. Reactions were performed at 30 °C for 40 min. Upper panels, the autoradiograph of the TLC; bottom panels, ceramidase activity. The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments. \textit{C16-Phyto-Cer}, \textit{C16-phytoceramide}; \textit{C16-Cer}, \textit{C16-ceramide}. 
gest that overexpression of YPC1p reverses fumonisin B1-induced growth inhibition by allowing synthesis of sphingolipids. To gain further insight into the function of YPC1p, we next examined the effect of overexpression of YPC1p on sphingolipid metabolism in the absence of fumonisin B1. Upon labeling with either palmitic acid (Fig. 3A) or serine (Fig. 3B), we saw the opposite effect from above. The strain Δyor1-YPC1 had a reduced rate of radiolabel incorporation into complex sphingolipids as compared with the strain Δyor1-Vec. On the other hand the Δyor1-YPC1 strain accumulated PHS, DHS, PHS-1-P, and DHS-1-P much more than the Δyor1-Vec (Fig. 3, A and B). These data raised an intriguing possibility that YPC1p has a dual function of enhancing synthesis or breakdown of sphingolipids, depending on the presence or absence of fumonisin B1.

**YPC1 Enhances Cellular Ceramidase Activity**—Next we set out to investigate the dual activity of YPC1p. Decreases in IPC and other complex sphingolipids and increases in DHS, PHS, and their phosphorylated products upon overexpression of YPC1p hinted that YPC1p might be mediating breakdown of either ceramides or more complex sphingolipids. We therefore investigated the ability of YPC1 to mediate breakdown of ceramides by labeling YPC1p-overexpressing cells with C-6-dihydroceramide labeled with 3H at both the C-4 and C-5 positions by labeling YPC1p-overexpressing cells with C-6-dihydroceramide labeled with 3H at both the C-4 and C-5 positions. We used C-6-dihydroceramide because it is taken up better than long acyl chain ceramides. Fig. 3C demonstrates that overexpression of YPC1p significantly enhanced degradation of dihydroceramide into DHS, PHS, DHS-1-P, and PHS-1-P. There were concomitant increases in phytosphingosine backbone. We used C-6-dihydroceramide as substrate, microsomes from the Δyor1-YPC1 cells were incubated at 30 °C for 90 min at pH 8.0. TLC was used to evaluate and quantitate product formation (Fig. 4A). When [3H]C-6-dihydroceramide was used as a substrate, microsomes from the Δyor1-YPC1 cells

![Fig. 7. YPC1 expressed in E. coli has ceramidase activity.](image)

**Fig. 7. YPC1 expressed in E. coli has ceramidase activity.** The His- and Xpress-tagged YPC1p expressed in E. coli Top10 cells was detected by Western blotting analysis using anti-Xpress antibody (A). Total soluble proteins were prepared from cells containing vector pBAD/His or pBAD/His-YPC1 and were applied to Talon metal affinity columns as described under "Materials and Methods." Proteins bound to the columns were eluted, and 20-μl eluants were assayed for ceramidase activity toward dihydroceramide (B) or phytoceramide (C) as described under "Materials and Methods." The data represent the mean of an experiment performed in duplicate and are representative of two independent experiments.

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**Fig. 8. YPC1 has ceramide synthase activity.** Microsomes from vector-containing cells (Δyor1-Vec) or YPC1p-overexpressing cells (Δyor1-YPC1) were added to phytosphingosine (5.0 nmol) and [9,10-3H]palmitic acid (10 μCi, 0.3 nmol) and incubated at 30 °C for 5 h. The products of the reaction were resolved by TLC and autoradiographed (A). Phytoceramide was identified according to a standard as described under "Materials and Methods" and was quantitated by a scintillation counter. Total activity was determined by subtracting background counts of the blank control (B). +Fum is the activity in the absence of fumonisin B1; -Fum is the activity in the presence of 50 μM fumonisin B1. The data represent the mean of an experiment performed in duplicate and are representative of four independent experiments.
YPC1 has the pH profile of an Alkaline Ceramidase Activity—In order to determine to which ceramidase category this enzyme belongs, we next determined its pH profile. We performed ceramidase activity from microsomal preparations of the YPC1p-overexpressing cells in different buffers ranging in pH from 4.5 to 10. Fig. 5 shows that at pH 5.0 or lower, YPC1 had hardly any ceramidase activity. The activity increased with increase in pH peaking at a pH of 9.5. These data suggest that YPC1 encodes an alkaline ceramidase activity.

YPC1 Increases Dihydro- and Phytoceramidase Activities but Not Unsaturated Ceramidase Activity—Yeast cells have only dihydro- and phytoceramides but no ceramide. As demonstrated in Fig. 4, YPC1p had dihydroceramidase activity. We next determined the substrate specificity of YPC1p ceramidase activity toward phytoceramide and unsaturated ceramide. We tested microsomal preparations from cells overexpressing YPC1p compared with microsomal preparations from vector cells using \(^{3}H\)-labeled C\(_{16}\)-phytoceramide and C\(_{16}\)-ceramide. Microsomes from the YPC1-overexpressing cells had significantly increased activity toward phytoceramide as compared with vector cells (Fig. 6A). Interestingly microsomes from the YPC1-overexpressing cells demonstrated no increase in ceramidase activity over the vector-containing cells when unsaturated ceramide was used as substrate (Fig. 6B).

Ceramidase Activity of YPC1 Expressed in E. coli—To confirm that YPC1p indeed was a ceramidase and not a regulator of cellular ceramidase activity, we proceeded to heterologously express YPC1p in E. coli. To facilitate detection and purification of YPC1p, we tagged it with the epitope tag polyhistidine and X-press in an expression vector pBAD/His B (Invitrogen). The expression of the tagged YPC1p was induced in LB medium with 0.2% arabinose for 4 h. The tagged YPC1p was purified by a His-tagged protein purification kit. To test if the purified YPC1p has ceramidase activity an in vitro assay of the activity was carried out with dihydroceramide and phytoceramide as substrates. Fig. 7, B and C, demonstrates that YPC1p expressed in E. coli had high ceramidase activity at pH 8.0 using either substrate, suggesting that YPC1p is indeed an alkaline ceramidase.

YPC1p is a Dual Activity Ceramidase and Palmitoyl-CoA-Independent Ceramide Synthase—Despite the discovery that YPC1p is a S. cerevisiae alkaline ceramidase, we still wanted to reconcile this with the fact that overexpression of YPC1p endowed fumonisin B1 resistance. Evidence that YPC1p overexpression allowed yeast cells to synthesize sphingolipids in the presence of fumonisin B1 prompted us to determine whether YPC1p also has ceramide synthase activity. To test this we again used microsomes from cells overexpressing YPC1p or containing the empty vector pYES2 and measured ceramide synthase activity by measuring ceramide formation using the same microsomal preparations as above with PHS and \(^{3}H\)palmitic acid as substrates. Fig. 8, A and B, demonstrates that microsomes prepared from cells overexpressing YPC1p had 10 times more ceramidase activity than did microsomes prepared from the \(\Delta yor1\)-Vec cells (Fig. 4B), thus indicating that YPC1p had ceramidase activity.

YPC1p is a Dual Activity Ceramidase and Palmitoyl-CoA-Independent Ceramide Synthase—Despite the discovery that YPC1p is a S. cerevisiae alkaline ceramidase, we still wanted to reconcile this with the fact that overexpression of YPC1p endowed fumonisin B1 resistance. Evidence that YPC1p overexpression allowed yeast cells to synthesize sphingolipids in the presence of fumonisin B1 prompted us to determine whether YPC1p also has ceramide synthase activity. To test this we again used microsomes from cells overexpressing YPC1p or containing the empty vector pYES2 and measured in vitro ceramide synthase activity. When we utilized DHS or PHS and palmitoyl-CoA as substrates with microsomes isolated from YPC1p-overexpressing cells, no increase in ceramide synthase activity was detected as compared with microsomes from vector cells (data not shown). This implied that YPC1p was not a CoA-dependent ceramide synthase. Other reports (8, 25) have indicated that ceramide can be synthesized from sphingosine and palmitic acid in a CoA-independent manner and may actually be a result of the “reverse” reaction of ceramidase. Formation of ceramide from palmitic acid, and not from palmitoyl-CoA, was shown to be catalyzed by a hypothetical reverse activity of a ceramidase from rat brain (30), human kidney and
cerebellum (31), guinea pig epidermis (32), and most recently by a bacterial ceramidase (8) or a sphingolipid ceramide deacylase (25). We next examined whether YPC1p encoded such an activity by measuring ceramide formation using the same microsomal preparations as above with PHS and \(^{3}H\)palmitic acid as substrates. Fig. 8, A and B, demonstrates that microsomes prepared from cells overexpressing YPC1p had 30-fold higher ceramide synthase activity; moreover, this increase in ceramide synthesis was not inhibited by the addition of fumonisin B1 to the in vitro reaction. These data suggest that YPC1p has an activity that catalyzes formation of ceramide from phytosphingosine and palmitic acid. This activity is distinct from the fatty acyl-CoA-dependent ceramide synthase activity that is inhibited by fumonisin B1. These data indicate that YPC1p is indeed a ceramidase with a reverse hydrolysis activity and prove the existence of such an enzyme.

Deletion of YPC1 Reduces Complex Sphingolipids—Finally, we wanted to evaluate phenotypes of the \(\Delta ypc1\) deletion mutant. We constructed a diploid strain with the \(\Delta ypc1\) null allele as described under “Materials and Methods.” Sporulation and tetrad dissection showed that deletion of YPC1 is viable, suggesting that the YPC1 gene is not an essential gene. In order to evaluate the endogenous function of YPC1p on metabolism of sphingolipids, we labeled the deletion mutant \(\Delta ypc1\) and its parental strain JK9–3d with \(^{3}H\)palmitic acid. TLC analysis demonstrated that all complex sphingolipids labeled by \(^{3}H\)palmitic acid increased in the \(\Delta ypc1\) strain compared with the wild type strain (Fig. 9). These data suggest that there is a block in the breakdown of these sphingolipids, presumably at the level of ceramidase. Ceramidase activity (hydrolyzing phytoceramide) from microsomal preparations of the \(\Delta ypc1\) strain was 1.4 \pm 0.2 units/mg as compared with 22.0 \pm 1.7 units/mg from the wild type strain JK9–3d. The CoA-independent (phasto-)-ceramide synthase activity from the \(\Delta ypc1\) strain was not detected as compared with 1.9 \pm 0.3 units/mg from the wild type strain. These data demonstrate that deletion of YPC1 significantly reduced (phasto-) ceramidase activity and obliterated CoA-independent (phasto-) ceramide synthase activity.

**FIG. 9.** Deletion of YPC1 enhances synthesis of complex sphingolipids. The same numbers (3 \(\times\) 10\(^5\)) of cells of the mutant strain \(\Delta ypc1\) and the parental strain JK9–3d \(\Delta yor1\) were labeled with \(^{3}H\)palmitic acid for 2 h as described in Fig. 2. Total lipids were extracted and resolved by TLC after hydrolysis by monomethylamine. The labeled sphingolipids are indicated.
Taken together, these results suggest that YPC1p indeed functions as an endogenous ceramidase in cells.

**DISCUSSION**

In this study, we describe the cloning of a novel *S. cerevisiae* gene YPC1 that endows resistance to the fungal toxin fumonisin B1. Our data firmly establish that the yeast gene YPC1 in fact encodes an alkaline ceramidase. First, YPC1p has an in vitro alkaline ceramidase activity with a pH optimum at pH 9.5. Second, YPC1p has substrate specificity toward yeast ceramides in that it catalyzes the breakdown of both phytoceramide and dihydroceramide but not mammalian unsaturated ceramide. Third, overexpression of YPC1p led to increased breakdown of labeled dihydroceramide and phytoceramide in intact yeast cells. Fourth, microsomes isolated from yeast cells that overexpress YPC1p had up to 30 times higher ceramidase activity than microsomes from cells that contain an empty vector. Finally, protein extracts from *E. coli* cells expressing YPC1p had significant increases in ceramidase activity as compared with extracts prepared from vector control bacterial cells. These data clearly indicate that YPC1 is indeed an alkaline ceramidase and not a regulator of the enzyme. We also demonstrate that YPC1p has the reverse activity that catalyzes the synthesis of yeast ceramides from palmitic acid and phytosphingosine or dihydrosphingosine. This ceramide synthase activity is coenzyme A-independent and occurs both in intact yeast cells and in vitro. This activity also explains why high copy expression of YPC1p endowed resistance to fumonisin B1.

Several different ceramidase activities have been described from eukaryotic (9, 15) and prokaryotic systems (8). They are classified as acidic, neutral, and alkaline ceramidases based on their pH optimum. Acid ceramidase was initially described from rat brain (30). At that time it was reported to have reverse catalytic activity. The acidic ceramidase is the enzyme whose inherited deficiency underlies ceramide accumulation in the lysosomal storage disease known as Farber’s disease (33). The enzyme was subsequently purified from human urine, cloned from human fibroblasts (11), and recently cloned from mouse tissue (12). It is localized in the lysosome, and it appears to prefer ceramides over dihydroceramides (11). However, there is no available evidence of the ability of the purified enzyme to catalyze the reverse reaction of ceramide synthase. Neutral ceramidase has been described from liver plasma membranes (34) and from rat intestinal brush border (35); however, not much is known about this enzyme. Recently, a membrane-bound ceramidase from rat brain was purified to homogeneity (15). It had an optimum pH in the neutral to alkaline range, appeared distinct from previously described ceramidases, and was termed non-lysosomal ceramidase. This enzyme also preferred unsaturated ceramide as substrate but was not evaluated for reverse catalytic activity. Alkaline ceramidase activity has been described from human cerebellum (31), fibroblasts (36, 37), and rat tissue (15, 34). In addition, two alkaline ceramidases were purified and characterized from guinea pig skin epidermis (32). Very recently an alkaline ceramidase was purified from the skin of patients with atopic dermatitis (8). This enzyme was from *Pseudomonas aeruginosa* that colonized skin; it required Ca$^{2+}$ for activity, and it hydrolyzed both ceramides and dihydroceramides equally but hydrolyzed phytoceramides less efficiently (8). Interestingly, this enzyme had the reverse activity of ceramide synthase. The *S. cerevisiae* alkaline ceramidase YPC1p appears to belong to this latter group of alkaline ceramidases. The ceramide synthase activity of YPC1p is distinguished from previously characterized ceramide synthesis activity by the following criteria. First, it is CoA-independent; second, it is not inhibited by fumonisin B1; and third, it prefers phytosphingosine and dihydrosphingosine.

YPC1p acts as a ceramidase or ceramide synthase, apparently depending on the availability of its substrate(s). In normally growing yeast cells, YPC1p overexpression led to the breakdown of phytoceramide or dihydroceramide. However, upon the addition of fumonisin B1 and consequent inhibition of endogenous yeast ceramide synthase, YPC1p was able to catalyze the synthesis of phytoceramide from palmitic acid and phytosphingosine both in cells and in vitro. In both yeast and mammalian cells, the synthesis of ceramide is mainly catalyzed by ceramide synthase that uses long chain bases and fatty acyl-CoAs as substrates and is inhibited by fumonisin B1. Identification of YPC1p as a dual activity enzyme indicates that a yet uncharacterized salvage pathway to synthesize ceramides exists in yeast. Therefore, YPC1p has a potentially important role in the synthesis of ceramide, especially when the CoA-dependent ceramide synthase is disabled. Importantly, the ability of this enzyme to catalyze ceramide synthesis and its lack of sensitivity to fumonisin B1 raises the possibility that fumonisin B1 is unable to inhibit totally ceramide synthesis.

Additionally, we have identified a protein encoded by a human expressed sequence tag that has very high homology to YPC1p. This homologue is likely to have dual activity, thus lending support to the notion that this salvage pathway for ceramide synthesis also exists in mammalian cells.

Regulation of both ceramide levels in yeast and mammalian cells is critical and must be carefully executed. On the one hand, ceramide is an essential building block for sphingolipids, whereby sufficient ceramide is needed for cell growth and viability (38, 39). On the other hand, ceramide is a stress sensor regulating many cellular responses including heat stress (18), growth arrest (18), and cell death (40). In addition cellular levels of sphingosine (the product of ceramidase) and sphingosine phosphate are critical for cellular well being in that they may mediate proliferation and oppose ceramide-induced apoptosis (41). YPC1p and its mammalian homologue, therefore, may be critically poised to readjust sphingolipid flow in cells depending on cellular levels of sphingosines and ceramides.

Deletion of YPC1 is viable and has no apparent phenotype but does affect endogenous metabolism of sphingolipids. As mentioned this gene has a homologue in *S. cerevisiae*. It remains to be evaluated if YPC1 and its homologue have similar functions. It is possible that deletion of both genes will be required for phenotype studies to be meaningful.

Identification of YPC1p as an alkaline ceramidase has advanced our knowledge of sphingolipid metabolism in the yeast *S. cerevisiae*. It is the first sphingolipid hydrolase to be identified in yeast. This affirms our belief that sphingolipid metabolic pathways are conserved between *S. cerevisiae* and higher eukaryotic species. These studies also underscore the importance of yeast genetic approaches to clone novel enzymes of sphingolipid metabolism.

**Acknowledgments**—We thank Dr. Yusuf Hannun for critical review of the manuscript and helpful discussions, Dr. George Farn (Biotechnology Resource Laboratory at Medical University of South Carolina) for assistance with DNA sequencing, and Dr. Samer El Bawab for assistance with the assay of ceramidase activity.

**REFERENCES**

1. Hannun, Y. A. (1996) *Science* **274**, 1855–1859
2. Hannun, Y. A., and Obeid, L. M. (1997) *Biochem. Soc. Trans.* **25**, 1171–1175
3. Spiegel, S., Cuvillier, O., Edsall, L. C., Kohama, T., Menzeleev, R., Olah, Z., Olivera, A., Pirianov, G., Thomas, D. M., Tu, Z., Van Brocklyn, J. R., and Wang, F. (1998) *Ann. N. Y. Acad. Sci.* **845**, 11–18
4. Dickson, R. C., and Lester, L. R. (1999) *Biochim. Biophys. Acta* **1438**, 305–321
5. Merrill, A. H., Jr., and Wang, E. (1992) *Methods Enzymol.* **200**, 427–437
6. Wu, W. I., McDonough, V. M., Nickels, J. T., Jr., Ro, J., Fischl, A. S., Vales, T. R., Merrill, A. H., Jr., and Carman, G. M. (1995) *J. Biol. Chem.* **270**, 13171–13178
7. Ito, M., Kurita, T., and Kita, K. (1995) *J. Biol. Chem.* **270**, 24370–24374
8. Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373.
9. Hassler, D. F., and Bell, R. M. (1993) Adv. Lipid Res. 26, 49–57.
10. Bernardo, K., Tani, M., Okino, N., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373.
11. Koch, J., Tani, M., Okino, N., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373.
12. Bernardo, K., Hurwitz, R., Zenk, T., Desnick, R. J., Ferlinz, K., Schuchman, E. H., and Sandhoff, K. (1995) J. Biol. Chem. 270, 11098–11102.
13. Koch, J., Gartner, S., Li, C. M., Quintern, L. E., Bernardo, K., Levran, O., Schuchman, E. H., and Sandhoff, K. (1996) J. Biol. Chem. 271, 33110–33115.
14. Li, C. M., Hong, S. B., Kopal, G., He, X., Linke, T., Hou, W. S., Koch, J., Gatt, S., Sandhoff, K., and Schuchman, E. H. (1998) Genomics 50, 267–274.
15. Nikolova-Karakashian, M., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 18718–18724.
16. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) J. Biol. Chem. 270, 11098–11102.
17. El Bawab, S., Bielawska, A., and Hannun, Y. A. (1999) J. Biol. Chem. 274, 27948–27955.
18. Saba, J. D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y. A. (1997) J. Biol. Chem. 272, 26087–26090.
19. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534.
20. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534.
21. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534.
22. Gietz, R. D., Schiestl, R. H. (1995) Methods Mol. Cell Biol. 5, 253–269.
23. Bielawska, A., Hanoun, Y. A. (1999) Methods Enzymol. 311, 499–518.
24. Mitsutake, S., Kita, K., Okino, N., and Ito, M. (1997) Anal. Biochem. 247, 52–57.
25. Mandala, S. M., Thornton, R. A., Frommer, B. R., Curotto, J. E., Rozdilsky, W., Kurtz, M. B., Giallef, R. A., Bills, G. F., Cabello, M. A., Martin, I., Peña, F., and Harris, G. H. (1995) J. Antibiot. (Tokyo) 48, 349–356.
26. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) J. Biol. Chem. 271, 10230–10236.
27. Beeler, T., Backova, D., Gable, K., Hopkins, L., Johnson, C., Slipe, H., and Dunn, T. (1998) J. Biol. Chem. 273, 30688–30694.
28. Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997) J. Biol. Chem. 272, 29704–29710.
29. Gatt, S. (1966) J. Biol. Chem. 241, 3724–3730.
30. Sugita, M., Williams, M., Dulaney, J. T., and Moser, H. W. (1975) Biochim. Biophys. Acta 398, 125–131.
31. Yada, Y., Higuchi, K., and Imokawa, G. (1995) J. Biol. Chem. 270, 12677–12684.
32. Spence, M. W., Reed, S., and Cook, H. W. (1986) Biochim. Biophys. Acta 814, 400–404.
33. Hertervig, E., Nilsson, A., Nyberg, L., and Duan, R. D. (1997) Cancer (Philad.) 79, 448–453.
34. Chatelut, M., Feunteun, J., Harzer, K., Fensom, A. H., Basile, J. P., Salvayre, R., and Levade, T. (1996) Cln. Chim. Acta 245, 61–71.
35. Momoi, T., Ben-Yosef, Y., and Nadler, H. L. (1982) Biochem. J. 205, 419–425.
36. Pinto, W. J., Wells, G. W., and Lester, R. L. (1992) J. Biol. Chem. 267, 2973–2989.
37. Hanada, K., Hara, T., Fukasawa, M., Yamaji, A., Umeda, M., and Nishi, M. (1998) J. Biol. Chem. 273, 33787–33794.
38. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Science 259, 1769–1771.
39. Cuvelier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) Nature 381, 800–803.