Cloning and Characterization of a *Saccharomyces cerevisiae* Alkaline Ceramidase with Specificity for Dihydroceramide*

Cungui Mao‡§, Ruijuan Xu‡§, Alicja Bielawska*, Zdzislaw M. Szulc†, and Lina M. Obeid‡¶

From the ‡Division of General Internal Medicine, Ralph H. Johnson Veterans Affairs Hospital and the ¶Departments of Medicine and Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29425

Received for publication, May 1, 2000, and in revised form, July 12, 2000

Published, JBC Papers in Press, July 18, 2000, DOI 10.1074/jbc.M003683200

In a previous study, we reported that the *Saccharomyces cerevisiae* gene YPC1 encodes an alkaline ceramidase with a dual activity, catalyzing both hydrolysis and synthesis of yeast ceramide (Mao, C., Xu, R., Bielawska, A., and Obeid, L. M. (2000) *J. Biol. Chem.* 275, 6876–6884). In this study, we have identified a YPC1 homologue in *S. cerevisiae* that also encodes an alkaline ceramidase. We show that these two ceramidases have different substrate specificity, such that YPC1p preferentially hydrolyzes phyto-ceramide, whereas the new ceramidase YDC1p hydrolyzes dihydroceramide preferentially and phytoceramide only slightly. Neither enzyme hydrolyzes unsaturated mammalian-type ceramide. In contrast to YPC1p, YDC1p had only minor *in vitro* reverse activity of catalyzing dihydroceramide formation from a free fatty acid and dihydrosphingosine and no activity with phytosphingosine. Overexpression of YDC1p had no reverse activity in non-stressed yeast cells, but like YPC1p suppressed the inhibition of growth by fumonisin B1 albeit more modestly. Deletion of YDC1 and YPC1 or both did not apparently affect growth, suggesting neither gene is essential. However, the Δydc1 deletion mutant but not the Δypc1 deletion mutant was sensitive to heat stress, indicating a role for dihydroceramidase but not phytoceramidase in heat stress responses, and suggesting that the two enzymes have distinct physiological functions.

Ceramide is a central molecule in the pathway of sphingolipid metabolism in mammalian cells (1, 2). It is converted through the action of a desaturase (3) from dihydroceramide that is synthesized *de novo* from dihydrosphingosine and a fatty acyl-CoA by (a CoA-dependent) ceramide synthase. Ceramide can also be generated from sphingomyelin by sphingomyelinase or be glycosylated to yield more complex glycosphingolipids. Ceramide is also broken down by ceramidases to generate sphingosine and a fatty acid.

Metabolism of yeast ceramides appears to be similar to that of mammals (4). Phytoceramide, an equivalent of the mammalian ceramide, serves as a building block of yeast complex sphingolipids. It is synthesized *de novo* from a fatty acyl-CoA and phytosphingosine by (a CoA-dependent) ceramide synthase. Phytosphingosine is generated from dihydrosphingosine through hydroxylation by the action of dihydrosphingosine hydroxylase (5, 6). Dihydrosphingosine is also acylated by a fatty acyl-CoA to generate dihydroceramide through the action of ceramidase synthase. Phytoceramide (or dihydroceramide) accepts an inositol-phosphoryl group to yield inositol phosphorylceramide (IPC).

Ceramide, as the building block of complex sphingolipids in eukaryotic cells, not only is structurally essential for cell growth but also is important in modulating different cellular events including apoptosis, growth arrest, and stress responses (see reviews Refs. 2 and 7–10). Importantly, its breakdown product sphingosine and the subsequent metabolite sphingosine-1-P are also signaling molecules (11). As a signaling molecule or a donor of signaling molecules, ceramide must be tightly regulated in order to carry out multiple functions in cells. Indeed, levels of ceramide in cells change in response to different physiological environments or to different stimuli including growth factors, cytokines (12), heat (4, 13), and pro-apoptosis agents (14). Changes in ceramide levels in cells involve different enzymes. Among these enzymes ceramidases are critical in controlling levels of ceramide in mammalian cells.

Several mammalian ceramidases have been purified, characterized biochemically, and subsequently cloned (15–17). They are classified as acid, neutral, and alkaline ceramidases according to their pH optimum. The acid ceramidase is localized to the lysosomes, and it is believed to be responsible mainly for housekeeping catabolism of membrane ceramide. Other membrane-bound ceramidases, which are considered as neutral- or alkaline-type enzymes, are believed to be in involved in signaling processes. For example, it was shown that mammalian alkaline ceramidase is activated by platelet-derived growth factor (12) and that both alkaline and neutral ceramidases are activated by interleukin 1β at a low concentration through tyrosine phosphorylation (18).

We recently cloned an alkaline ceramidase YPC1p from *Saccharomyces cerevisiae* (19). YPC1p preferentially deacylated phytoceramide to yield a free fatty acid and phytosphingosine. It also slightly deacylated dihydroceramide to generate a free fatty acid and dihydrosphingosine, but it did not act on unsaturated ceramide. Importantly, this alkaline ceramidase had a reverse activity of catalyzing formation of phytoceramide from
a free fatty acid and phytosphingosine in vitro and in cells. Formation of phytoceramide by the reverse activity of YPC1p appears to be an important alternative pathway for the synthesis of phytoceramide when the Coa-dependent ceramide synthase is inhibited in S. cerevisiae. Identification of YPC1p suggests that breakdown of ceramides is conserved between yeast and mammals.

Another alkali ceramidase was recently cloned from Pseudomonas and mycobacterium (20). This enzyme also had reverse activity but had no sequence homology to the yeast enzyme YPC1p, indicating they belong to different classes of ceramidas. In fact there was no homologous yeast sequence to the Pseudomonas ceramidase. Interestingly the Pseudomonas alkali ceramidase shared a similarity in protein sequence and many biochemical properties with the mouse neutral ceramidase, which was recently purified and cloned from mouse liver (16, 21) and a human mitochondrial ceramidase cloned from human kidney (22).

On the other hand, a search of protein data bases for homology to YPC1p identified that another yeast putative protein encoded by the open reading frame YPL087w has 53% identity to YPC1p. In this report, we present evidence that this protein (YDC1p) is another membrane-bound alkaline ceramidase, which was recently purified and cloned from S. cerevisiae (18). The YDC1p coding region and the reverse primer (underlined) encoding the FLAG epitope peptide by PCR was amplified by PCR (1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; 1 cycle of 72 °C for 10 min) using the primers 5'-CGGGGTACCCATGCTGTTCAGCTGGCCTTATCCAG-3' and the reverse primer 5'-CGGAATTCGTTATCTTCTTTTGGTCTACATACC CGGTTATGTTTGTTTGTTATCCAG 3'.

Preparation of Radiolabeled Ceramides—[3H]Ceramide and phyto-sterol ceramide in S. cerevisiae Alkaline Dihydroceramidase YDC1p 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 (24).
β-Erythrosphingosine was obtained in stereo- and enantio-specific synthesis as described previously (25). Phytosphingosine was from Sigma. N-Hexanoyl-β-erythro-[4,5-3H]dihydrosphingosine ([3H]C6-dihydroceramide), β-erythro-[4,5-3H]dihydrosphingosine, and N-erythro-[4,5-3H]dihydrosphingosine-1-phosphate were from American Radiolabeled Chemicals (ARC, Inc.).

Measurements of Ceramidase Activity and Its Reverse Activity—Ceramidase activity was measured using [3H]ceramide (2.75 nmol), [3H]phytoceramide (2.5 nmol), or [3H]C6-dihydroceramide (2 nmol) as a substrate as described (19). To measure ceramidase activity, 20 μl (approximately 150 μg of proteins) of microsomes or a purified protein was added to substrates, and reactions were incubated at 30 °C for 40–90 min. Reactions were stopped by adding 300 μl of methanol: chloroform (2:1) and dried under a SpeedVac. Lipids were resolved by TLC; the reaction product palmitic acid or dihydrosphingosine was identified, scraped, and measured by a scintillation counter. Both the protein concentration and the time of incubation were within the linear range for the assay. One unit of ceramidase was defined as the amount of the enzyme needed to release 1 pmol of palmitic acid or dihydrosphingosine per min.

The reverse activity of ceramidase was measured using [3H]palmitic acid (0.3 nmol) and phytosphingosine (or dihydrosphingosine) (5 nmol) as substrates as described (19). Microsomes were added to the above substrates and incubated at 30 °C for 2 h. Both protein concentration and time of incubation were within the linear range for the assay. Phospholipid determination on microsomes showed that different microsomal preparations with equal amount of proteins contained equal amount of lipids. One unit of the reverse activity was defined as the amount of the enzyme needed to form 1 pmol of phytoceramide (or dihydroceramide) per min.

Sphingolipid Labeling—Cells (3 × 10^7 in 1 ml of medium) were labeled with [3H]palmitic acid or [3H]C6-dihydroceramide (5–10 μCi) at 30 °C for different periods as described (19). 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, 9:7:2, v/v) as described (19). TLC plates were sprayed with the solvent system II (chloroform, methanol, 4.2 N ammonium hydroxide, 9:7:2, v/v) as described (19). TLC plates were sprayed with EN^2HANCE and radiographed on BioMax films (Eastman Kodak Co.). Radiolabeled sphingolipids were identified according to authentic standards. 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-PAGE and were detected by Coomassie staining or Western blotting analysis by following standard procedures.

Heat Tolerance Study—Exponentially growing cells of yeast strains were incubated at 50 °C for 40 min. The heat-treated cells and those set at room temperature were plated onto YPD plates and incubated at 30 °C for 2–3 days. The plates were photographed and colony-forming units (CFU) were determined. The post-heat stress viability was defined as a percentage of post-heat stress CFU to those prior to heat stress.

RESULTS

Identification of Another Yeast Ceramidase—To find potential homologues for the YFCI gene, we searched the Saccharomyces Genome Database. We identified a putative protein, encoded by the open reading frame YPL087w, that had 52% identity to the yeast alkaline ceramidase YDCIp over the entire protein sequence. This protein had 317 amino acids, with a predicted pl of 6.77. Several highly conserved regions were found between the two proteins (Fig. 1A). Similar to YPC1p, the homologue was a very hydrophobic protein and shared a similar hydropathy profile with YPC1p (Fig. 1B). Both proteins were predicted to have several transmembrane domains (Fig. 1C), suggesting that this homologue is also an integral membrane protein. Both proteins had an ER retention sequence (KKXX, X represents any amino acid residue) at their carboxyl termini, suggesting that they may be localized to the ER. Based on the sequence, the protein sequence and hydropathy profiles, this protein was predicted to be another ceramidase.

To investigate whether the homologous gene encodes for a ceramidase activity, we cloned its coding sequence into the vector pYES2 under the control of the Gal1 inducible promoter. Sequencing confirmed that the coding region was identical to that reported in the yeast genomic data base. The vector pYES2 and the new construct were transformed into the yeast strain Δyor1, and gene expression was induced by galactose. Microsomes were prepared and assayed for ceramidase activity using different ceramides as substrates. Interestingly overexpression of the homologous protein caused a substantial increase in ceramidase activity toward C6-dihydroceramide (Fig. 2A), but only a slight increase in the activity toward phytoceramide.
Fig. 2. YDC1p hydrolyzes dihydroceramide preferentially and phytoceramide only slightly but does not hydrolyze saturated ceramide. Microsomes prepared from cells overexpressing YDC1p or YPC1p were assayed for ceramidase activity toward dihydroceramide (A), phytoceramide (B), or unsaturated ceramide (C) as described under “Experimental Procedures.” The upper panels show the TLC separation of products from substrates, and the bottom panels show ceramidase activity. Data are the mean of one experiment performed in duplicate and are representative of at least three independent experiments. C6-dh-cer, C6-dihydroceramide.

(Fig. 2B), and no activity toward unsaturated ceramide (Fig. 2C). In contrast to the homologue, microsomes from cells overexpressing YPC1p hydrolyzed phytoceramide preferentially over dihydroceramide (Fig. 2, A and B). These data suggest that this homologue also encodes for a ceramidase activity; however, it had different substrate specificity. We thus renamed this gene YDC1 as yeast dihydroceramidase.

Tagged and Purified YDC1 Encodes a Ceramidase Activity—We demonstrated above that YDC1p encodes a ceramidase activity mainly hydrolyzing dihydroceramide. However, we
have not ruled out the possibility that YDC1p is a regulator of ceramidase activity. To verify that YDC1p is itself a ceramidase, we went on to express the YDC1p in *E. coli*. To facilitate detection and purification, we tagged the YDC1p with a polyhistidine (His) tag. The His-tagged YDC1p was expressed in *E. coli* as analyzed by Western blot (data not shown). The His-tagged YDC1p did not have ceramidase activity, probably due to a post-translational modification required for the activity that *E. coli* lacks. Therefore, we elected to express the YDC1p in yeast cells. To facilitate purifying the YDC1p, we tagged it with an epitope tag FLAG. Expression of the tagged YDC1p was induced by galactose after the expressing construct pYES2-YDC1-FLAG was introduced into yeast cells. Microsomes were prepared from the cells expressing YDC1-FLAG or containing the empty vector pYES2-FLAG. Proteins were extracted from microsomes with 0.25% Triton X-100 and applied to an anti-FLAG affinity column. After washing, the FLAG-tagged protein was eluted by a buffer containing FLAG peptide (10 μg/ml). The eluates were resolved by SDS-PAGE, and proteins were detected by Coomassie staining (Fig. 3A) and Western blot analysis (Fig. 3B) using the anti-FLAG antibody. A protein band with an apparent molecular mass of 37 kDa and a cluster of protein bands with apparent molecular mass ranging from 75 to 200 kDa were revealed by Coomassie staining as well as by Western blotting in the YDC1-FLAG eluate but not in the vector control eluate. The eluates were assayed for ceramidase activity. Fig. 3C shows that the purified FLAG-tagged YDC1p, but not the vector control eluate, had ceramidase activity toward C6-dihydroceramide. The high molecular weight protein cluster could be aggregated YDC1-FLAG since it is a very hydrophobic protein. Alternatively, it could be YDC1-FLAG associated complexes. However, since YDC1p is highly homologous to the alkaline ceramidase YPC1p, and purified YDC1p had ceramidase activity toward C6-dihydroceramide, it is most likely that the YDC1 gene encodes a ceramidase and less likely that it is a regulator of enzymatic activity.

YDC1p Is Also an Alkaline Ceramidase—In our previous study, we demonstrated that YPC1p is an alkaline ceramidase, with an optimal pH of 9.5 (19). To study whether YDC1p has the same or a different pH optimum, we measured the ceramidase activity of microsomal preparations from cells overexpressing YDC1p using C6-dihydroceramide as a substrate at different pH. Fig. 4 shows that YDC1p has very low activity at acidic pH, moderate activity at neutral pH, and the highest activity at alkaline pH. These data suggest that similar to YPC1p, YDC1p also belongs to the alkaline ceramidase family.

YDC1p Functions as a Ceramidase in Cells—*In vitro* YDC1p showed ceramidase activity. We next wanted to know whether in cells YDC1p has the same ceramidase activity. Ceramidase activity in cells was evaluated using [3H]C6-dihydroceramide labeled at the C-4 and C-5 positions of the dihydrosphingosine moiety. Similar to YPC1p, overexpression of YDC1p enhanced breakdown of [3H]C6-dihydroceramide, thus leading to accumulation of dihydrosphingosine (DHS), phytosphingosine (PHS), DHS-1-P, PHS-1-P, and the glycerolipids phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (Fig. 5), suggesting that YDC1p has endogenous ceramidase activity in cells.

YDC1p Has the Reverse Activity of Synthesizing Dihydroceramide from a Fatty Acid and Dihydrosphingosine—To investigate if the YDC1p has this reverse activity similar to its homologue YPC1p, microsomes prepared from cells containing the empty vector (pYES2) or overexpressing YDC1p were assayed for the reverse activity. Microsomes were incubated with [3H]palmitic acid and phytosphingosine or palmitic acid and dihydrosphingosine at 30 °C for 2 h. The product, phytocera-
control cells (Fig. 6). Cells had 3-fold higher reverse activity than those from vector
palmitic acid were used as substrates. Microsomes from YDC1 from YDC1p-overexpressing cells had no increase in the reverse activity. When dihydrosphingosine and phytosphingosine and palmitic acid were used as substrates. We wondered whether in the presence of fumonisin B1, overexpression of YDC1 could have the reverse activity in cells. Cells containing pYES2-YDC1, the vector, or pYES2-YPC1 (as a positive control) were grown on SC-ura plates containing 450 μM fumonisin B1. Fig. 7A shows that YDC1p also endowed resistance to fumonisin B1 albeit somewhat less than YPC1p. This result shows that YDC1p could have the reverse activity of ceramidase in cells under stress conditions but was not as effective as YPC1p.

Fumonisin B1 induced cytotoxicity in yeast could be due to blockage of synthesis of ceramides and complex sphingolipids, or due to accumulation of long chain bases and their phosphates, or both. We have previously shown that a large proportion of exogenous long chain bases were phosphorylated by long chain base kinases to yield long chain base phosphates that are toxic to yeast cells (26). YPC1p or YDC1p could endow resistance to fumonisin B1 by using accumulated long chain bases to synthesize the yeast ceramides and attenuate the cytotoxicity of the long chain base phosphates. To test this, we evaluated if overexpression of either ceramidase could endow resistance to the long chain bases phytosphingosine and dihydrosphingosine by diverting them to phytoceramide and dihydroceramide using the reverse activity. The strains containing the vector pYES2, pYES2-YPC1, and pYES2-YDC1 were spotted onto SC-ura plates containing 25 μM phytosphingosine and 2% galactose, and growth inhibition of the different strains was examined by the serial cell dilution method described above. Fig. 7B shows that the YPC1 strain is more resistant to phytosphingosine than the vector strain, whereas the YDC1 strain is only slightly more resistant to phytosphingosine, suggesting that the YPC1 strain, and to a much lesser extent, the YDC1 strain using its reversal action of ceramidase, converts phytosphingosine to phytoceramide in cells when phytosphingosine is in excess. This result is consistent with the in vitro study, such that YPC1p, but not YDC1p, can use phytosphingosine as substrate to synthesize yeast ceramide. On the other hand, neither the vector strain nor the YPC1 or YDC1 strains were sensitive to DHS (up to 70 μM). This is compatible with our other data indicating that PHS but not DHS mediates yeast growth arrest.

Deletion of Both YDC1 and YPC1 Eliminates All Basal Ceramidase Activity toward Phytoceramide and Dihydroceramide—To study the physiologic functions of YPC1p and YDC1p, we made deletion mutants of YPC1, YDC1, or both. First, we examined how deletion of these two genes affects metabolism of sphingolipids. We labeled the mutants and their parental strain with [3H]palmitic acid and analyzed total sphingolipids by TLC. Fig. 8 shows that deletion of either YPC1 or YDC1 caused an increase in IPC, MIPC, and M(IP)2C and a decrease in DHS-1-P and PHS-1-P compatible with their function as ceramidases. Deletion of both YPC1 and YDC1 had an additive effect on metabolism of sphingolipids. We speculated that complex sphingolipids increase in the deletion mutants because ceramidase activity in these mutants is significantly decreased or totally abolished. Therefore, we measured ceramidase activity of these mutant strains. Microsomes prepared from these

---

*Fig. 4. YDC1p has the highest activity toward dihydroceramide at pH 9.5–10. Microsomes prepared from cells overexpressing YDC1p were assayed for the activity of hydrolyzing dihydroceramide at different pH values. Sodium acetate buffer was used for pH 4.5–6; Tris-HCl buffer was used for pH 7–8. Glycine HCl buffer was used for pH 9–10.5. Data are the mean of one experiment performed in duplicate and are representative of at least three independent experiments.**

*Fig. 5. Overexpression of YDC1p or YPC1p causes an increase in breakdown of C₆-dihydroceramide, thus leading to accumulation of free long chain bases and their phosphates. Cells containing the vector (pYES2), expressing YPC1p (pYES2-YPC1), or YDC1p (pYES2-YDC1) were labeled with [3H]C₆-dihydroceramide as described under “Experimental Procedures.” Total lipids were extracted and resolved by TLC, and sphingolipids were identified according to authentic standards. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.*

---

N. Chung, C. Mao, J. Heitman, Y. A. Hannun, and L. M. Obeid, manuscript in preparation.
Strains and assayed for ceramidase activity (Table II) showed that deletion of YPC1 eliminated most of the basal ceramidase activity for phytoceramide but slightly reduced the activity for dihydroceramide. In contrast, deletion of YDC1 eliminated most of the activity toward dihydroceramide but only slightly reduced the activity toward phytoceramide. However, deletion of both YPC1 and YDC1 completely removed the activity toward both phytoceramide and dihydroceramide. These data suggest that YPC1p and YDC1p indeed have their respective substrate specificity in cells and are the only enzymes responsible for catabolism of the yeast ceramides (dihydroceramide and phytoceramide).

Deletion of Both YDC1 and YPC1 Eliminates Most of the Reverse Activity of Ceramidase—We also measured reverse activity of ceramidase in the deletion mutants. Table III shows that deletion of YPC1 eliminated most of the reverse activity when either phytosphingosine or dihydrosphingosine was used as substrates. Deletion of YDC1 showed no change in the activity when phytosphingosine and palmitic acid were used as substrates but showed slight reduction in the activity when dihydrosphingosine and palmitic acid were used as substrates. Deletion of both YPC1 and YDC1 knocked out most of the activity when either phytosphingosine or dihydrosphingosine were used as substrates along with palmitic acid. These results suggest that the reversal action of ceramidase in yeast cells is mainly carried out by YPC1p and to a much lesser extent by YDC1p.

Both YPC1p and YDC1p Are Localized to ER—Both YPC1p and YDC1p have an ER retention sequence, suggesting they may be localized to the ER. We examined their localization by tagging YPC1p and YDC1p with a green fluorescent protein (GFPuv). Western blotting analysis using an anti-GFP antibody was performed on extracts from cells expressing GFPuv, GFPuv-YPC1p, or GFPuv-YDC1p fusion proteins as described under “Experimental Procedures.” Fig. 9A shows that free GFPuv was detected only in the 100,000 × g supernatant, whereas both GFPuv-YPC1p and GFPuv-YDC1p were detected only in the 100,000-g pellet, suggesting that both YPC1p and YDC1p are membrane proteins as predicted, and their localization in cells was not affected by GFPuv tagging. The fluorescent pattern of exponentially growing cells expressing the GFPuv-YPC1p or YDC1p fusion was recorded by a CCD camera. Fig. 9B shows that GFPuv-tagged YPC1p and YDC1p have a typical ER pattern shared by another lipid enzyme DHS-1-P phosphatase YSR2, suggesting they are indeed localized to the ER where most enzymes of sphingolipid metabolism are localized.

Deletion of YDC1, but Not YPC1, Causes a Decrease in Tolerance to Heat Stress—Yeast cells deficient in YPC1p, YDC1p, or both are viable and have normal growth rates in both rich medium and defined medium under permissive temperatures, suggesting that neither YPC1p nor YDC1p is essential. The yeast ceramides and other sphingolipids have been implicated in the response to heat stress, whereby their levels are elevated in heat-stressed cells (13, 27). Increased ceramides were suggested to come from the de novo pathway (27). We previously showed that elevation of dihydroceramide or phytoceramide imparted on yeast cells a sensitivity to heat stress, whereas elevation of dihydrosphingosine-1-P has been implicated in heat resistance (26). Deletion of either YPC1 or YDC1 caused an increase in synthesis of complex sphingolipids due to decreased breakdown of ceramides. To study whether the pathway of breakdown of ceramides is involved in the process of heat tolerance, we examined the presence or absence of fumonisin B1 (Fum). The top panel shows the TLC profile of lipids, and the bottom panel shows the reverse activity. Data are the mean of one experiment performed in duplicate and are representative of at least three independent experiments.
stress, we investigated heat stress responses in the mutants deficient in YPC1p, YDC1p, or both. Cells were incubated at 50 °C for 40 min, and post-heat viability was determined by CFU on YPD plates. Fig. 10 shows that the post-heat stress viability of both the Δydc1 and Δypc1Δydc1 mutant strains was lower than that of either the Δypc1 mutant or the wild type strains. The Δypc1 strain that lacks the ability to break down phytoceramide had the same response as the wild type JK9-3d to heat. On the other hand, the Δydc1 that lacks the ability to break down dihydroceramide was highly sensitive to heat stress. The Δypc1Δydc1 strain, like Δydc1, was also more sensitive to heat stress. These results suggest that accumulation of dihydroceramide but not phytoceramide may be responsible for mediating sensitivity to heat stress. Alternatively, loss of breakdown product in the Δydc1 strain (dihydrosphingosine), but not in the Δypc1 strain (phytosphingosine), could be responsible for mediating this heat sensitivity.

**DISCUSSION**

In this study, we identified and cloned a homologue to the *S. cerevisiae* phytoceramidase (YPC1p). We demonstrate that this homologue is also an alkaline ceramidase that hydrolyzes dihydroceramide specifically, and we named it yeast dihydroceramidase (YDC1p). YDC1p shows reverse activity of ceramidase *in vitro*; however, unlike YPC1p, YDC1p has a minor reverse activity and only with dihydrosphingosine as substrate. In cells, YDC1p acts as a ceramidase under normal culture conditions, but the ceramidase action can be reversed in the presence of fumonisin B1. Both YPC1p and YDC1p have an ER retention sequence at their carboxyl termini, and we verify their localization to the ER by GFP tagging. We also demonstrate that deletion of YDC1 but not YPC1 renders cells sensitive to heat stress, suggesting that dihydroceramide (or dihydrosphingosine), but not phytoceramide (phytosphingosine), in yeast may have a distinct role in response to heat stress.

Several ceramidases have been cloned from mammalian cells. These include an acid lysosomal ceramidase (15), a mouse liver neutral ceramidase (16), and its homologue a human kidney mitochondrial ceramidase (17). These latter two enzymes are homologous to the *Pseudomonas* alkaline ceramidase (20). We have also recently identified and cloned a human

**FIG. 7.** Overexpression of YPC1p rescues from growth inhibition by both fumonisin B1 and phytosphingosine, whereas overexpression of YDC1p rescued from fumonisin B1 only. Cell cultures were serially diluted and spotted onto SC-ura plates with 2% galactose, containing fumonisin B1 (A), phytosphingosine (B), or neither fumonisin B1 nor phytosphingosine, incubated at 30 °C for 3 days, and photographed by an imaging system (Alpha Innotech Inc).

**FIG. 8.** Deletion of YPC1, YDC1, or both affects metabolism of sphingolipids in cells. Cells were labeled with palmitic acid, and total lipids were extracted and resolved by TLC after base hydrolysis as described under "Experimental Procedures." Sphingolipids were identified according to authentic standards. JK9-3d, the wild type strain.
homologue of the yeast alkaline ceramidases. Comparison of protein sequence revealed that our yeast ceramidases and their human homologue are completely distinct from the acid lysosomal ceramidase as well as the mouse neutral ceramidase and the human mitochondrial ceramidase, which in turn are also distinct from the acid ceramidase. Therefore, based on protein sequence, it is not yet possible to predict a substrate-binding site.

As far as substrate specificity, the yeast ceramidases prefer yeast-saturated ceramides as their substrates, whereas all the other neutral and alkaline ceramidases described to date prefer unsaturated ceramide (21, 22). The bacterial ceramidase also uses mammalian type unsaturated ceramide as substrate; therefore, the enzyme may have a role in bacterial invasiveness. In fact such a role was raised in the study of Ohnishi et al. (28) where the Pseudomonas ceramidase was implicated in hydrolyzing skin ceramides in atopic dermatitis. Therefore, whether these different ceramidases have distinct physiologic roles needs to be further studied.

Protein motif prediction using the PROSITE program revealed that both YPC1p and YDC1p have putative cAMP protein kinase tk;1phosphorylation sites and protein kinase C phosphorylation sites, and YDC1p has a tyrosine kinase phosphorylation site (Fig. 1A). It has been shown that activity of mammalian neutral and alkaline ceramidases is regulated by protein phosphorylation. Whether the yeast ceramidases are phosphorylated and the phosphorylation modifies their enzymatic activity awaits further study.

It is believed that ceramides are synthesized in the ER. Localization of YPC1p and YDC1p to the ER suggest that YPC1p and YDC1p have immediate access to the yeast ceramide as soon as it is formed. The benefit of the rapid access of these enzymes to the ceramides could be to regulate turnover of ceramides most efficiently because ceramide levels are crucial for the well being of yeast cells. YDC1p and YPC1p are the only ceramidases in yeast cells, so the ER should be an important pool for generating dihydrophosphingosine and phytosphingosine, the products of these enzymes. We previously showed that dihydrophosphingosine-1-P phosphatases (YSR2 and YSR3) are also localized to the ER. These data suggest that the ER is the center of both synthesis and breakdown of yeast sphingolipids.

In mammalian cells, it is believed that sphingosine is derived from deacylation of ceramide by ceramidase and not from de novo biosynthesis. Its subsequent metabolite sphingosine-1-P is also one of the breakdown products of ceramide. Sphingosine and sphingosine-1-P have been implicated in opposite cellular actions, such that sphingosine suppresses cell growth and sphingosine-1-P promotes cell proliferation. In yeast, sphingo-

### Table II

| Ceramidase | WT | Δypc1 | Δydcl | Δypc1 Δydcl |
|------------|----|-------|-------|-------------|
| Phytoceramidase activity (units/mg) | 19.8 ± 0.5 | 1.2 ± 0.4 | 19.0 ± 1.0 | 0.76 ± 0.2 |
| Dihydroceramidase activity (units/mg) | 5.4 ± 0.8 | 4.87 ± 0.3 | 0.51 ± 0.1 | 0.31 ± 0.1 |

### Table III

| Strain | WT | Δypc1 | Δydcl | Δypc1 Δydcl |
|--------|----|-------|-------|-------------|
| Phytoceramidase synthase activity (units/mg) | 15.3 ± 0.4 | 0.88 ± 0.1 | 14.9 ± 0.5 | 0.77 ± 0.3 |
| Dihydroceramide synthase activity (units/mg) | 9.8 ± 0.6 | 1.1 ± 0.18 | 7.4 ± 0.8 | 0.6 ± 0.1 |

FIG. 9. Both YPC1p and YDC1p are localized to ER. YDC1p and YPC1p were tagged with a fluorescent GFPuv as described under “Experimental Procedures.” Expression of the tagged proteins was induced in SC-ura medium containing 2% galactose. Cells were disrupted by glass bead collision, and whole cell lysate was fractionated into cytosolic and membrane fractions after removal of nuclei. Proteins from the two fractions were separated by SDS-PAGE, GFPuv, and the GFPuv fused YPC1p and YDC1p were detected by Western blot analysis using anti-GFP antibody (A). Fluorescence of cells was examined under a fluorescent microscope and recorded by a digital camera (B).
Deletion of phosphatase (YSR2) also sensitizes cells to heat stress (26). We demonstrated that overexpression of dihydrosphingosine-1-P dihydroceramide but not phytoceramide is responsible for the respective increase in dihydroceramide and a decrease in dihydrosphingosine-1-P. Thus, we conclude that changes in dihydroceramide, dihydrosphingosine-1-P, or both are responsible for the heat stress sensitivity.

Acknowledgment—We thank Dr. Yusuf Hannun for critical review of the manuscript and helpful discussions.

REFERENCES
1. Luberto, C. H. Y. (1999) Lipids 34, S–11
2. Merrill, A. H., Jr., Schmeizl, E. M., Dillehay, D. L., Spiegel, S., Shyma, J. A., Schroder, J. J., Riley, R., T. Voss, K. A., and Wang, E. (1997) Toxicol. Appl. Pharmacol. 142, 208–225
3. Michel, C., van Richten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 22432–22437
4. Dickson, R. C., and Lester, R. L. (1999) Biochim. Biophys. Acta 1438, 305–321
5. Grilley, M. M., Stock, S. D., Dickson, R. C., Lester, R. L., and Takemoto, J. Y. (1998) J. Biol. Chem. 273, 11062–11068
6. Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997) J. Biol. Chem. 272, 29704–29710
7. Hannun, Y., and Obeid, L. M. (1997) Biochem. Soc. Trans. 25, 1171–1175
8. Hannun, Y., and Obeid, L. M. (1997) Adv. Exp. Med. Biol. 407, 145–149
9. Hannun, Y. A. (1997) Adv. Exp. Med. Biol. 305–312
10. Hannun, Y. A. (1996) Science 274, 1855–1859
11. Spiegel, S., Cuvillier, O., Edsall, L. C., Kohama, T., Menzelev, R., Ohla, 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
12. Coronel, E., Martinez, M., McKenna, S., and Kester, M. (1995) J. Biol. Chem. 270, 23305–23309
13. Jenkins, G. M., Richards, A., Wahl, T., Mao, C., Obeid, L., and Hannun, Y. (1997) J. Biol. Chem. 272, 32566–32572
14. Luci, A., Han, Y. T., Liu, Y. Y., Giuliano, A. E., and Cabot, M. C. (1999) Int. J. Oncol. 15, 541–546
15. Koch, J., Gartner, S., Li, C. M., Quintern, L. E., Bernardo, K., Levrari, O., Schnabel, D., Desnick, R. J., Schuchman, E. H., and Sandhoff, K. (1996) J. Biol. Chem. 271, 33110–33115
16. Tani, M., Okino, N., Mori, K., Tanigawa, T., Ito, H., and Ito, M. (2000) J. Biol. Chem. 275, 11229–11234
17. El Bawab, S., Bielawska, A., Babiak, Z., Piotrowska, A., and Hannun, Y. (2000) J. Biol. Chem. 275, 21508–21513
18. Nikolova-Karakashian, M., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 18178–18172
19. Mao, C., Xu, S., Bielawska, A., and Obeid, L. M. (2000) J. Biol. Chem. 275, 6876–6884
20. Okino, N., Ichinose, S., Omori, A., Imayama, S., Nakamura, T., and Ito, M. (1999) J. Biol. Chem. 274, 36616–36622
21. Tani, M., Okino, N., Mitsutake, S., Tanigawa, T., Ito, H., and Ito, M. (2000) J. Biol. Chem. 275, 3462–3468
22. El Bawab, S., Bielawska, A., and Hannun, Y. A. (1999) J. Biol. Chem. 274, 27848–27855
23. Mao, C., Wadleigh, M., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1999) J. Biol. Chem. 274, 28690–28694
24. Bielawska, A., and Hannun, Y. A. (1999) Methods Enzymol. 311, 499–518
25. Bielawska, A., and Hannun, Y. A. (1999) Methods Enzymol. 311, 518–547
26. Mao, C., Suba, J. D., and Obeid, L. M. (1999) Biochem. J. 342, 667–675
27. Wells, G. B., Dickson, R. C., and Lester, R. L. (1996) J. Biol. Chem. 273, 7235–7243
28. Ohnishi, Y., Okino, N., Ito, M., and Imayama, S. (1999) Clin. Diag. Lab. Immunol. 6, 101–104