Involvement of Yeast Sphingolipids in the Heat Stress Response of Saccharomyces cerevisiae

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A role for sphingolipids in the yeast heat stress response has been suggested by the isolation of suppressors of mutants lacking these lipids, which are unable to grow at elevated temperatures. The current study examines the possible role of sphingolipids in the heat adaptation of yeast cells as monitored by growth and viability studies. The suppressor of long chain base auxotrophy (SLC, strain 7R4) showed a heat-sensitive phenotype that was corrected by transformation with a serine palmitoyltransferase. Thus, the deficiency in sphingolipids and not the suppressor mutation was the cause of the heat-sensitive phenotype of the SLC strain 7R4. The ability of sphingolipids to rescue the heat-sensitive phenotype was examined, and two endogenous yeast sphingoid backbones, phytosphingosine and dihydrosphingosine, were found to be most potent in this effect. Next, the effect of heat stress on the levels of the three major classes of sphingolipids was determined. The inositol phosphoceramides showed no change over a 1.5-h time course. However, the four detected species of sphingoid bases increased after 15 min of heat stress from 1.4- to 10.8-fold. The largest increases were seen in two sphingoid bases, C20 phytosphingosine and C20 dihydrosphingosine, which increased 6.4- and 10.8-fold over baseline, respectively. At 60 min of heat stress two species of yeast ceramide increased by 9.2- and 10.6-fold over baseline. The increase seen in the ceramides was partially decreased by Fumonisin B1, a ceramide synthesis inhibitor. Therefore, heat stress induces accumulation of sphingoid bases and of ceramides, probably through de novo synthesis. Taken together, these results demonstrate that sphingolipids are involved in the yeast heat stress adaptation.

Saccharomyces cerevisiae has been shown to respond to a transfer of 25–37 °C with the physiology defined as a heat stress response (1, 2), which appears to involve two phases. The initial phase of the response is the gaining of thermotolerance, and an increase in trehalose accumulation is proposed as a marker for this event (3). This is accompanied by the induction of heat shock proteins (4) and a G1 arrest in cell cycle that lasts for a period of approximately 1 h (5). Once thermotolerance is gained, the second phase of the response occurs when the yeast begin to grow at the increased temperature. At this point, trehalose is degraded in an HSP70-dependent process (6), and the cells begin to cycle and resume growth. Therefore, the ability of yeast to grow under increased temperature provides an overall assessment of the heat stress response. However, the mechanisms that mediate adaptation and growth under the heat-stressed state are not fully defined.

The isolation of suppressors of mutants lacking sphingolipids in yeast (Table I) has suggested a possible role for sphingolipids in the heat stress response. The initial mutation is a Ura disparity knockout of the serine palmitoyltransferase (SPT) gene (LCB1) (7), which catalyzes the first step of sphingolipid biosynthesis (Fig. 1). Consequently, the 134 strain lacks all sphingolipids. This yeast strain (134) is unable to grow at normal temperatures without sphingoid backbone supplementation to the media (7). Suppressors (7R4 and 4R3) of the aforementioned mutant have been isolated, and were found to allow growth in the absence of sphingolipid supplementation, possibly as a result of the formation of novel inositol glycerolipids. The novel inositol glycerolipids contain: 1) the 26 carbon fatty acid that is ordinarily exclusively found in the yeast sphingolipids; and 2) the same head groups that are found in the inositol phosphoceramides, now attached to a glycerol backbone (8, 9). Thus, these sphingolipid compensatory strains are able to grow at normal temperatures but with a slower doubling time than the wild-type strain (SJ21R). However, the SLC1 strains still lack all sphingolipids. The SLC strains were found not to grow under conditions of cellular stress such as high osmolality and increased ambient temperature. However, the heat-sensitive phenotype could be reversed by the addition of phytosphingosine to the media (10).

The rescue of the SLC lines via phytosphingosine indicates a possible need for sphingolipids in the yeast heat stress response. The current study examines the role of sphingolipids in the heat adaptation of yeast cells. First, the SLC strain 7R4 was transformed with the serine palmitoyltransferase gene to ensure that the observed heat-sensitive phenotype was due to the lack of sphingolipids and not to the secondary mutation. Next the specificity of sphingolipids that allowed for growth during heat stress was examined. Furthermore, the levels of sphingoid backbones, ceramides, and the inositol phosphoceramides (IPC) were measured in response to heat stress. Our results show that endogenous sphingolipid levels change in response to a heat stress challenge and that sphingolipids are necessary for the heat stress response of yeast.

EXPERIMENTAL PROCEDURES

Chemicals and Compounds—Sphingosine, phytosphingosine, dihydrosphingosine, and fumonisins B1 were from Sigma. 1-Threo-Dihy-
drosphingosine, C2 phytoceramide, C2 and C6 ceramides, and C2 and C6 dihydroceramides were synthesized as described previously (11, 12).

**Yeast Strains—** Yeast strains used are all isogenic to the wild-type strain SJ21R. Strain 1D4 is the URA disruption of the serine palmitoyltransferase gene (lcb1). Suppressors of the long chain base knockout (SLC's) used in this study were the 4R3 and 7R4 strains, and they were obtained from Dr. Robert Dickson (University of Kentucky) (7, 8). The strain 7R4-LCB1 was created for this study as described below.

**Yeast Transformation—** The plasmid PLCB1–5 was a gift from Dr. Robert Dickson. The LCB1 gene in the plasmid was cut at its unique SmaI site at base 263 and EcoRI site at base 936 to obtain a 573-base pair fragment with which to transform the yeast strain 7R4. The transformation was done using the lithium acetate protocol (13). To select for the desired recombination event, the transformed yeast were plated on 5-fluoroorotic acid. Controls for transformation efficiency were streaked on leucine-deficient plates. Transformation results were confirmed by polymerase chain reaction.

**Plate Growth Studies—** Yeast strains were streaked and then grown for 2 to 4 days at 37 °C. The plated strains were then photographed and manually scored for growth. Plates containing supplementary sphingolipids were made with or without detergent Nonidet P-40 used as a dispersant at a concentration of 0.05%.

**Liquid Growth Studies—** Yeast strains were seeded at $2 \times 10^5$ to $8 \times 10^5$ cells/ml and assayed for growth under the given conditions and treatments. At the selected time points, a 1-ml aliquot was taken out, and its optical density was measured at 600 nm.

**Lipid Extraction—** Yeast sphingolipids were extracted from liquid cultures via the method of Bligh and Dyer (14). The resultant lipids were resuspended and split into three thirds. One-third was for organic phosphates, which were used to normalize samples to each other. The other two-thirds were used for the measurement of sphingoid bases and ceramide measurements.

**Sphingosine Measurements—** The resultant lipid extract was analyzed after derivatization with ortho-phthalaldehyde via HPLC with a Shimadzu fluorescent detector (15). Samples were compared by use of the unnatural sphingoid base L-threo-dihydrosphingosine as an internal standard.

**FIG. 1.** Biosynthetic pathway of yeast sphingolipids. The biosynthesis of sphingolipids is shown in the above proposed pathway.

**FIG. 2.** Growth of 7R4-LCB1 at both 30 and 39 °C. The strains SJ21R (wild type), 7R4 (suppressor of lcb1) and 7R4-LCB1 (suppressor and LCB1) were streaked out on YEPD (yeast extract, bacto-Peptone, dextrose) plates. One plate was grown at 30 °C and the other at 39 °C for 2 days.

**FIG. 3.** Effects of phytosphingosine and dihydrosphingosine on growth at 39 °C of the SLC strain 7R4. Phytosphingosine (psph) (A) or dihydrosphingosine (dhsph) (B) were added a half hour before the zero time point of heat stress (39 °C) at the given final concentrations. Growth was assayed by absorbance at 600 nm of a 1-ml aliquot for the indicated time points. Each point is the average of two separate measurements shown with their standard deviation.

| Yeast strains |
|----------------|
| Strain | Genotype | Reference |
| SJ21R | MATa, ura3–52, leu2–3, 112, ade1, MEL1 | 7 |
| 1D4 | MATa, leu2–3, 112, ade1, MEL1, lcb1::URA3 | 7 |
| 4R3 | MATa, leu2–3, 112, ade1, MEL1, lcb1::URA3, SLC1–1 | 8 |
| 7R4 | MATa, leu2–3, 112, ade1, MEL1, lcb1::URA3, SLC2–1 | 8 |
| 7R4-LCB1 | MATa, ura3–52, leu2–3, 112, ade1, MEL1, SLC2–1 | This study |
standard added before the Bligh and Dyer extraction.

Ceramide Measurements—Lipid extracts were phosphorylated by *Escherichia coli* diacylglycerol kinase (16). The phosphorylated compounds were then separated on a thin layer chromatography (TLC) plate and then exposed to film. For quantification, the plates were subsequently exposed to a phosphoimager screen. The screen was then scanned, and analysis of the resulting image was performed via Image Quant.

Inositol Phosphoceramides Measurements—Cells in liquid culture were labeled with tritiated inositol overnight and then treated as described (17). Yeast lipid extracts were prepared and run on TLC plates as described (17). The resultant TLC plates were exposed to film to obtain a permanent image. Quantification was done via Image Quant. An aliquot for the measurement of organic phosphate was taken before the lipids were run on TLC plates. The resultant phosphate value was used to normalize the values obtained via Image Quant.

RESULTS

Necessity for Sphingolipids in the Heat Stress Response in *S. cerevisiae*—The suppressor of auxotrophy for long chain bases, 7R4, displays a heat-sensitive phenotype (see Fig. 2). To ensure that this phenotype is not a result of the compensatory mutation in the strain 7R4, we reinserted the deleted *LCB1* gene. A 573-base pair *LCB1* gene fragment was generated from the plasmid PLCB1–5 by separate digestion with *Sma* and *EcoRI*, and the fragment was used to transform cells via lithium acetate. Colonies were selected for growth on 5-fluoroorotic acid. This selection requires the yeast to have lost the *URA* gene disruption of the endogenous *LCB1* through homologous recombination to grow. Thus, the selected strain should contain this *SPT* gene under its normal regulation. Eleven colonies from two transformations were identified. Seven of these were selected for further study. The seven selected transformants did not grow upon Ura<sup>−</sup> plates (data not shown), providing further evidence that the gene had been properly inserted and had essentially deleted the *URA* gene via recombination. Finally, polymerase chain reaction was done with two sets of primers, and the selected mutant 7R4 2B (7R4-LCB1) was found to have the expected products (data not shown). Strain 7R4-LCB1 was tested for its ability to grow at both 30 and 39 °C (Fig. 2). The 7R4 strain grew moderately at 30 °C and was not able to grow at all at 39 °C (Fig. 2). On the other hand, the 7R4-LCB1 strain grew as well as the wild-type SJ21R at both temperatures. At 30 °C, acquisition of sphingolipids by the strain via the reinsertion of this *SPT* gene under its normal control allowed for wild-type growth as compared with the slower growth of the 7R4 strain. These data indicate that indeed the slow growth of the 7R4 strain is due to its lacking of sphingolipids. Furthermore, the ability of 7R4-LCB1 to grow as well as the wild type at 39 °C demonstrates that the secondary mutations did not impart the heat-sensitive phenotype seen in the 7R4 strain. Therefore, these data prove that the lack of sphingolipids causes the heat stress-deficient phenotype seen in the SLC strain, and, thus, the presence of sphingolipids is
Specificity of Sphingolipids in the Rescue of the Heat Stress-deficient Phenotype—The necessity of sphingolipids for SLC growth at high temperature led to studies on the ability and specificity of various mammalian and yeast sphingolipids to allow for this growth. These studies were performed in solid and liquid media.

The growth of the SLC strains was studied in liquid culture where a quantitative assessment of the ability of sphingolipids to increase growth was obtained. The heat-sensitive phenotype of the SLC strains 7R4 and 4R3 was overcome by both phyto-sphingosine and dihydrosphingosine (Figs. 3 and 4). The growth of the SLC strain 7R4 was most potently increased by 1 mM dihydrosphingosine. However, 1 and 5 mM phyto-sphingosine also allowed for heat-stressed growth of the 7R4 strain (Fig. 3). In the SLC strain 4R3, 1 and 5 mM phyto-sphingosine were more effective than 1 mM dihydrosphingosine in allowing for heat-stressed growth of the SLC strain 4R3 (Fig. 4). The heat-sensitive phenotype of the SLC strain 7R4 was not reversed by the addition of 1 or 5 mM d-erythro-sphingosine or C2 phyto-ceramide (data not shown). Furthermore, d-e-sphingosine could not rescue the SLC strain 4R3. Therefore, the rescue of the SLC strains from their heat-sensitive phenotype was accomplished only by the two endogenous yeast sphingoid backbones phyto-sphingosine and dihydrosphingosine.

The growth studies on plates supported the findings of those found in the liquid studies. The plate growth data at 37 °C showed that phyto-sphingosine was the most potent long chain base in allowing for growth with heat stress (data not shown). However, the other endogenous sphingoid backbone, d-erythro-dihydrosphingosine also allowed for SLC growth at 37 °C. In addition C2 phytoceramide, d-e-sphingosine, C5 and C6 ceramide along with C2 and C5 dihydroceramide did not show an ability to overcome the heat-sensitive phenotype of the 7R4 and 4R3 SLC strains (data not shown). Overall, the plate and liquid studies showed that only yeast endogenous sphingoid backbones allowed for growth at heat-stressed temperatures.
Stress—The necessity of sphingolipids in the heat stress response and the ability of the endogenous sphingoid backbones to allow for growth at high temperature prompted us to examine the levels of endogenous sphingolipids in the wild-type cells upon heat stress. Each of the three major classes of sphingolipids was measured over a given time course of heat stress.

The first class of sphingolipids measured in *S. cerevisiae* were the inositol phosphoceramides. Liquid cultures were labeled overnight with tritiated inositol. Lipids from heat-stressed and nonheat-stressed cells were extracted by a previously described method (17), and the resultant extracts were developed on TLC plates (Fig. 5A). Over a 1.5-h time course of heat stress, these data showed no significant changes in IPC, mannose IPC, and mannose (inositol phosphate)$_2$ ceramide.

**Figure 7.** Effects of heat stress on levels of ceramides. Extracted lipids (see Ref. 14) were phosphorylated by diacylglycerol kinase and run out on thin layer chromatography plates. A, film of the TLC plate with lanes corresponding to the following: lanes 1 and 2, zero time point; lanes 3 and 4, 15 min control; lanes 5 and 6, 15 min heat stress; lanes 7 and 8, 30 min control; lanes 9 and 10, 30 min heat stress; lanes 11 and 12, 60 min control; and lanes 13 and 14, 60 min heat stress. B, ceramide 1 control versus heat stress levels. C, ceramide 2 control versus heat stress levels. Each measurement is the average of duplicates displayed with their standard deviation. Data are representative of more than three experiments.
bands (Fig. 5B). Furthermore, no changes were seen in phosphati-dylinositol and lysophosphatidylinositol (data not shown).

The second class of sphingolipids measured were the sphingo-goid backbones (long chain amino bases). The sphingoid backbones from liquid cultures were first extracted under Bligh and Dyer conditions. The organic phase was dried down and resuspended, and an aliquot was taken to measure the organic phosphate measurement. The remaining extract was put through a mild base hydrolysis and reextracted. The resultant lipids were dephosphorylated with phthaldialdehyde and separated via high performance liquid chromatography using a C18 reverse phase column. The extraction efficiency of each sample was evaluated by the use of a nonendogenous sphingoid backbone, d-erythro-dihydrosphingosine, added prior to the initial Bligh and Dyer extraction. Four peaks on the HPLC charts were identified as sphingoid bases. Four criteria were met in order for these peaks to be identified as such. The first was the resistance to mild base hydrolysis. Second, these two peaks were designated C20 phytosphingosine and C20 dihydrosphingosine due to their longer retention times. Each time point measured was compared with a time-matched nonheat-stressed control (Fig. 6C). Within 15 min of heat stress at 39 °C there was a large induction of the sphingoid backbones (Fig. 6D). At the 15-min time point, C18 phytosphingosine increased 1.4-fold, whereas C18 d-erythro-dihydrosphingosine had increased 2.2-fold over the time matched controls (Fig. 6D). The largest increases were seen in the C20 sphingosine and C20 dihydrosphingosine species. C20 sphingosine increased 6.4-fold over baseline (Fig. 6, C and D). Also, at 15 min C20 dihydrosphingosine increased 10.8-fold over its time-matched control (Fig. 6D). These increases were transient in that the levels decreased to near baseline by 1 h (Fig. 6D). The 7R4-LCB1 line showed this phenomena, as did another wild-type yeast line W303 (data not shown). Overall, the sphingoid backbones showed large and transient increases with heat stress in several yeast lines.

The third class of sphingolipids measured were the yeast ceramides. The lipids from liquid cultures were extracted by the method of Bligh and Dyer. After resuspension, an aliquot was taken for organic phosphate measurement. The remaining lipids were phosphorylated by E. coli diacylglycerol kinase and run out on TLC plates. We found two bands of ceramide phosphates via TLC separation. These two bands were identified as ceramides due to their absence in the SLC lines and resistance to mild alkaline hydrolysis (data not shown). The ceramides increased following 60 min (Fig. 7) of heat stress and continued to be elevated until at least 2 h of heat stress (Fig. 8A). As shown, ceramide 1 increased by 9.2-fold (Fig. 7B), and the ceramide 2 increased by 10.6-fold (Fig. 7C). A smaller increase of 2.7-fold in diacylglycerol was also seen after an hour of heat stress at 39 °C (data not shown). The increase seen in the ceramides was partially inhibited by a 2-h preincubation period with 150 μM fumonisin B1 (Fig. 8, A and B). Fumonisin B1 has been shown to inhibit the ceramide synthase in yeast (see Fig. 1) (18). Therefore, these data indicate that the increased ceramide derives at least partially from the conversion of sphingoid backbones to ceramides.

The above data show that the sphingoid backbone species undergo a large and transient increase upon heat stress. Also, ceramides show a large increase with later kinetics and appear sustained for at least 1 h. This increase occurred after the return of the sphingoid backbones to basal levels. Furthermore, the results with fumonisin B1 indicate a conversion of the sphingoid backbones to ceramides in the heat stress response.

**FIG. 8. Effect of fumonisin B1 on heat stress-induced increase of ceramides.** Extracted lipids (see Ref. 14) were phosphorylated by diacylglycerol kinase and run out on thin layer chromatography plates. A, film of the TLC plate with lanes corresponding to the following: lane 1, diacylglycerol standard; lanes 2 and 3, 60 min controls; lanes 4 and 5, 60 min heat stress; lanes 6 and 7, 60 min heat stress pretreated with 150 μM fumonisin B1 for 2 h; lanes 8 and 9, 120 min controls; lanes 10 and 11, 120 min heat stress; and lane 12 mammalian ceramide control. B, comparison of ceramide 1 and ceramide 2 at 60 min with and without heat stress and with heat stress preincubated with 150 μM fumonisin B1 for 2 h before heat stress. Each measurement is the average of duplicates displayed with their standard deviation.

**DISCUSSION**

The question of the need for sphingolipids in the heat stress response has arisen due to the isolation of suppressor mutations of the knockout of serine palmitoyltransferase in yeast (7). These SLC mutants are unable to grow under many states of cellular stress including increased ambient temperature, unless the media they grow upon is supplemented with phytosphingosine (10). Therefore, we set out to determine whether the observed phenotype of heat sensitivity is due to a lack of sphingolipids or due to the secondary suppressor mutations. As shown, the 7R4-LCB1 strain with a reinserted serine palmitoyltransferase gene (LC1B) is not heat sensitive. Thus the heat sensitivity did not arise from the aforementioned suppressor mutations. Therefore, we conclude that sphingolipids are necessary for the yeast heat stress response.

The measurement of sphingolipids in heat-stressed wild-type yeast showed dramatic increases in two classes of sphingolipids. Within 15 min of heat stress all four species of sphingoid backbones increased with the C20 phytosphingosine and C20 phytosphingosine and C20 dihydrosphingosine were identified as such, whereas the remaining two peaks were designated C18 phytosphingosine and C18 dihydrosphingosine due to their longer retention times. Each time point measured was compared with a time-matched nonheat-stressed control (Fig. 6C). Within 15 min of heat stress at 39 °C there was a large induction of the sphingoid backbones (Fig. 6D). At the 15-min time point, C18 phytosphingosine increased 1.4-fold, whereas C18 d-erythro-dihydrosphingosine had increased 2.2-fold over the time matched controls (Fig. 6D). The largest increases were seen in the C20 sphingosine and C20 dihydrosphingosine species. C20 sphingosine increased 6.4-fold over baseline (Fig. 6, C and D). Also, at 15 min C20 dihydrosphingosine increased 10.8-fold over its time-matched control (Fig. 6D). These increases were transient in that the levels decreased to near baseline by 1 h (Fig. 6D). The 7R4-LCB1 line showed this phenomena, as did another wild-type yeast line W303 (data not shown). Overall, the sphingoid backbones showed large and transient increases with heat stress in several yeast lines.

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dihydrosphingosine each showing over 6-fold increases. Furthermore, the increases seen were transient, and levels returned to baseline by 1 h. Yeast ceramides also increased but not until 1 h of heat stress and remained elevated for at least an additional hour. The increases seen in the ceramides were partially inhibited by fumonisin B1, indicating that at least part of the increase was from the conversion of yeast sphingobackbones to ceramide via ceramide synthase. Furthermore, the increase in ceramides could be inhibited nearly completely by Australifungin, a potent and specific inhibitor of ceramide synthase. Moreover, for up to 1.5 h of heat stress, the IPCs did not significantly change. Taken together these data suggest enhanced de novo synthesis of these compounds, which could be the source of the increased sphingoid bases and subsequently the increased ceramides.

One can speculate on the role of sphingolipids in the heat stress response. There are two prominent possibilities. The first possibility is that the role played by sphingolipids is of a structural nature. This possibility is based upon the observation that membrane IPC’s comprise about 30% of the phospholipid content of plasma membrane in yeast (19). Therefore, the reason that SLC strains may acquire sensitivity to heat could be due to the lack of these sphingolipids in the membranes. One piece of evidence that indicates that this might not be the case is provided by the novel inositol glycerolipids. These compounds are very similar to the IPCs in structure and are hypothesized to fulfill their structural functions. This idea is supported by the data that the SLC strains will grow at normal temperatures, whereas the serine palmitoyltransferase knock-out will not. However, the novel inositol glycerolipids may not totally mimic the function of wild-type sphingolipids. The second hypothesis suggests that the sphingolipids play a more regulatory role that goes beyond their structural role. This is supported by the observed changes in sphingolipid levels following heat stress. These results raise the possibility that yeast sphingolipids react to heat stress and may participate in the adaptive responses, possibly as second messengers or signal transducers. Such a role has been proposed in mammalian systems where sphingolipids have been found to participate in senescence (20), differentiation (21, 22), apoptosis (23), and cellular stress responses (24). In addition mammalian cells have been shown to display an increase in ceramide in response to heat stress and that ceramide induces the expression of at least one heat shock protein, α B crystallin (25). In light of these roles in mammalian systems one can easily theorize that yeast sphingolipids may play a similar role in the yeast heat stress response.

The results from the growth studies show that only endogenous yeast sphingoid backbones are able to rescue the SLC cells from their inability to grow in an increased ambient temperature. These data indicate a possible importance for the sphingoid backbones in the yeast heat stress response. In light of this idea one can speculate that the yeast sphingoid backbones, which show major increases upon heat stress, are important in a signaling role of this stress response. Second, the increase in ceramides may also play a role. Indeed, these biphasic changes in yeast sphingolipids may reflect the complex physiology of the yeast heat stress response, which may be considered as a biphasic physiology. First the yeast tries to survive the new temperature. Once this is accomplished the yeast may then regain its ability to grow. Questions such as what are the roles of sphingoid backbones and ceramides in the yeast heat stress response should be addressed. Also, one would like to delineate the necessity of either or both of the above sphingolipids in the gaining of thermotolerance and regrowth phases of heat stress. Finally, if there is indeed a signaling role for sphingolipids in the heat stress response, the study of where and how these molecules affect the heat stress response should be quite interesting.

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