Role for \textit{de Novo} Sphingoid Base Biosynthesis in the Heat-induced Transient Cell Cycle Arrest of \textit{Saccharomyces cerevisiae}*

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The recent findings of sphingolipids as potential mediators of yeast heat stress responses led us to investigate their possible role in the heat-induced cell cycle arrest and subsequent recovery. The sphingolipid-deficient yeast strain 7R4 was found to lack the cell cycle arrest seen in the isogenic wild type. Furthermore, strain lcb1-100, which harbors a temperature-sensitive serine palmitoyltransferase, lacked increased \textit{de novo} generated sphingoid bases upon heat stress. Importantly, this strain was found to lack the transient heat-induced G\textsubscript{s}/G\textsubscript{1} arrest. These results indicate a role for sphingolipids and specifically those generated in the \textit{de novo} pathway in the cell cycle arrest response to heat. To determine the bioactive sphingolipid regulating this response, an analysis of key mutants in the sphingolipid biosynthetic and degradation pathways was performed. Strains deleted in sphingoid base kinases, sphingoid phosphate phosphatase, lyase, or dihydro sphingosine hydroxylase were found to display the cell cycle arrest. Also, the knockout of a fatty acyl elongation enzyme, which severely attenuates ceramide production, displayed the arrest. These experiments suggested that the active species for cell cycle arrest were the sphingoid bases. In further support of these findings, exogenous phytosphingosine (10 \textmu M) was found to induce transient arrest. Stearylamine did not induce an arrest, demonstrating chemical specificity, and 1-erythro- was not as potent as 1-\textit{erythro}\textit{-}dihydrosphingosine showing stereospecificity. To investigate a possible arrest mechanism, we studied the hyperstable Cln3 (Cln3–1) strain LDW6A that has been previously shown to be resistant to heat-stress-induced cell cycle arrest. The strain containing Cln3–1 was found to be resistant to cell cycle arrest induced by exogenous phytosphingosine, indicating that Cln3 acts downstream of the sphingoid bases in this response. Interestingly, cell cycle recovery from the transient arrest was found to be dependent upon the sphingoid base kinases (LCB4, LCB5). Overall, this combination of genetic and pharmacologic results demonstrates a role for \textit{de novo} sphingoid base biosynthesis by serine palmitoyltransferase in the transient G\textsubscript{s}/G\textsubscript{1} arrest mediated through Cln3 via a novel mechanism.

\textit{Saccharomyces cerevisiae} has been shown to respond to an increase in temperature from 30 to 39 °C with a physiology known as the heat stress response (1, 2), which involves two phases. The initial phase is the gaining of thermotolerance through an accumulation of trehalose (3), an induction of the heat shock proteins (4), and a transient arrest of the cells in the G\textsubscript{s}/G\textsubscript{1} phase of the cell cycle (5). The transient cell cycle arrest is characterized as a decrease in budding after 1 h of heat stress (6). The decrease in budding has been shown to be blocked by expression of a hyperstable Cln3, indicating a role for G\textsubscript{s} cyclins in this response (7). Furthermore, G\textsubscript{s} cyclins are regulators of entry into S phase at the point known as START, and, upon heat stress, transcript levels of Cln1 and Cln2 are decreased (7). A decrease in available G\textsubscript{s} cyclins induces a G\textsubscript{s}/G\textsubscript{1} arrest. After thermotolerance has been achieved, the second phase of the heat stress response is characterized by a resumption of normal growth at the elevated temperature. This phase is marked by a HSP-70-dependent process of trehalose degradation (8) and resumption of a normal cell cycle as seen by a recovery of budding by 2 h of heat stress (6). The mechanisms involved in regulating the cell cycle response to heat are not yet defined.

Examination of suppressor mutants of the lethal deletion of serine palmitoyltransferase subunit (\textit{LCB1}) has begun to shed light on the roles of sphingolipids in the yeast heat stress response (9–11). The suppressor makes novel inositol glycerolipids that mimic the inositol phosphoceramides, thus allowing for growth under normal conditions (12, 13). However, under heat stress and other stress conditions, the suppressor mutants were found to be defective in growth as compared to the isogenic wild type yeast strain (14). Thus, the study of these mutants resulted in the finding that sphingolipids are necessary for the yeast heat stress response (9, 11).

The induction of \textit{de novo} synthesis of free sphingoid bases and yeast ceramides upon heat stress have suggested a role for these lipids as possible signaling moieties. Already, two such functions have been attributed to sphingolipids in the yeast heat stress response. First, addition of exogenous sphingolipids was shown to induce a reporter gene attached to a stress response element (10). Second, it was shown that sphingoid bases mediate a ubiquitin-dependent degradation of nutrient permeases in response to heat stress (15).

The current study examines the role of sphingolipids in the heat-induced transient cell cycle arrest. An initial role for sphingolipids was disclosed by experiments on the sphingolipid deficient strain 7R4. Also, the temperature-sensitive serine palmitoyltransferase strain lcb1-100 was found to be defective in the transient cell cycle arrest, indicating a requirement for \textit{de novo} generated sphingolipids in this effect. The role of specific sphingolipids in the cell cycle arrest was investigated using knockouts of sphingolipid enzymes, and these results implicate the free sphingoid bases as the bioactive species. A

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\textit{The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s).}

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yeast strain containing the hyperstable Cln3–1 showed this cyclin to act downstream of the sphingoid base-induced arrest. Finally, a role of the sphingoid base kinases in cell cycle recovery was determined, and is suggested to likely be through clearance of the sphingoid bases rather than the formation of the phosphorylated sphingoid bases per se. These data show that de novo synthesis of sphingoid base is involved in the transient cell cycle arrest likely through Cln3 and that recovery from the cell cycle arrest is partially mediated by the sphingoid base kinases.

MATERIALS AND METHODS

Chemicals, Compounds, and Yeast Strains—\(\text{-}\)erythro-Sphingosine, phytosphingosine, RNase, proteinase K, and stearylamine were obtained from Sigma. Both \(\text{-}\)erythro- and \(\text{-}\)erythro-dihydrosphingosine were from Avanti. Tritiated palmitate and tritiated palmitoyl-CoA were obtained from American Radiolabeled Chemicals. Propidium iodide was obtained from American Radiolabeled Chemicals. Yeast strains used in this study are listed in Table I with genotype.

Budding Determination—The percentage of budded cells upon heat stress was determined by seeding 1–2 \(\times\) 10^6 cells/ml in 5 ml of yeast extract, bactopeptone and dextrose (YPED) media. Cells were resuspended in ~0.5 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol, and a 5 mM concentration of chloroform:methanol (1:2, v/v). Dihydrosphingosine (50 \(\mu\)M) was added as carrier. To induce the phase break, 1 ml of chloroform and 1 ml of 0.5 N ammonium hydroxide were added and the solution was vortexed. The aqueous phase was discarded, and the organic phase was dried down. The organic extract was then resuspended in chloroform, spotted on a plate for thin layer chromatography (TLC), and developed in a solvent system of chloroform:methanol:2 N ammonium hydroxide (40:10:1, v/v). The plate was then sprayed with EnHANCE and exposed to film. The spot comigrating with keto-dihydrosphingosine was scraped and counted. A blank of no protein was extracted and run as a background.

Labeling Cells with Palmitate—Two milliliters of YEPD media was seeded with 5 \(\times\) 10^9 cells/ml and rested for 2 h. Tritiated palmitate (1 \(\mu\)Ci/ml) was added, and the cells were incubated for 6 h. Cells were pelleted, washed with water, and extracted as described (17). The lipids were dried down and base-hydrolyzed as described previously (18). The resulting lipids were dried down and resuspended in 75 \(\mu\)l of chloroform:methanol:water (1:2:0.1, v/v) and spotted onto a TLC. The TLC plate was developed in a solvent system of chloroform:methanol:4.2 N ammonium hydroxide (9:7:2, v/v). Results were imaged by spraying with ENHANCE and exposing to film for 3 days.

Extraction and Analysis of Sphingoid Bases—A total of 2 \(\times\) 10^6 cells were taken from an overnight YEPD culture. Extraction was done as described previously via a modified Bligh and Dyer extraction (19, 19). Lipids were then dried down, and two-thirds were used for sphingoid measurement with the other one-third used for organic phosphate measurement. The lipids for sphingoid base measurements were derivatized with ortho-phthalaldialdehyde and separated over a reverse phase C18 column with detection by a Shimadzu fluorescent detector essentially as described (20). Samples were compared by use of the nonendogenous sphingoid base \(\text{-}\)threo-dihydrosphingosine as an internal standard added before the lipid extraction.

Cell Cycle Studies—Yeast cells were seeded at 5 \(\times\) 10^9 to 2 \(\times\) 10^6 cells/ml in YEPD media and rested 6–8 h at 30 °C. Yeast cells were then treated under the given conditions of temperature and/or lipid concentration. At the given time points a control and treated sample were taken and centrifuged. The cell pellet was washed twice with ice cold water and then fixed in 1 ml of 70% ethanol, all at 4 °C. After fixing overnight, the cells were again pelleted, washed with 5 ml of 50 mM sodium citrate, and then resuspended in 1 ml of sodium citrate. RNase was then added and incubated for 1 h at 50 °C, and then proteinase K was added and incubated under the same conditions. Finally, 0.85 ml of sodium citrate and 0.15 ml of propidium iodide solutions were added. Cells were kept 24 or more h in the dark at 4 °C. Cells (15,000) were analyzed per histogram using a Becton Dickinson fluorescence-activated cell analyzer. Data were modeled using the program ModFit LT.

RESULTS

Requirement for Sphingolipids in Heat-induced Transient Cell Cycle Arrest—The role of sphingolipids in the heat-induced transient cell cycle arrest was investigated in the sphingolipid-deficient strain, 7R4, and its isogenic wild type 7R4-LCB1. Strain 7R4-LCB1 showed the expected arrest at 1 h for 39 °C as.
heat stress. Yeast cells of strain 7R4-LCB1 (photographed at 100×) were pelleted, resuspended, and placed on slides. A representative field was photographed at 100× and scored for budding. Each bar is the average of two separate measurements shown with their range.

evaluated by budding (Fig. 1A). Budded 7R4-LCB1 cells decreased from 66% in the asynchronous samples to 24% at 1 h of heat stress and recovered to 62% budded by 2 h of heat stress. In contrast, the sphingolipid-deficient strain 7R4 did not arrest. Strain 7R4 had 58% budded cells in an asynchronous culture and upon 1 h at 39 °C maintained 60% budded cells, with 55% of cells still budded at 2 h (Fig. 1B). Because the only difference between these two strains is the presence or absence of sphingolipids, these results show that lack of sphingolipids results in an inability to transiently arrest upon heat stress.

_de novo_ Formation of Sphingolipids through Serine Palmitoyltransferase Is Necessary for Heat-induced Transient G<sub>0</sub>/G<sub>1</sub> Arrest—To investigate the role of _de novo_ sphingolipid synthesis in the heat-induced arrest, strain lcb1-100 was characterized in terms of serum palmitoyltransferase activity, production of sphingolipids at 30 °C, and increased _de novo_ synthesis of sphingoid bases upon heat stress. This temperature-sensitive strain, lcb1-100, was isolated as a secretory mutant (end8-1) containing a point mutation in the _LCB1_ gene (21), which is a necessary component of serum palmitoyltransferase (22). Proteins from the strains lcb1-100, RH406, 7R4-LCB1, and 7R4 were extracted to compare the _in vitro_ serine palmitoyltransferase activity. Protein extract from strain 7R4-LCB1 produced 2.88 nmol of keto-dihydrosphingosine per milligram of protein, and protein extract from strain RH406 produced 1.52 nmol of keto-dihydrosphingosine per milligram of protein (Fig. 2A). Protein extract from the _LCB1_ deletion strain 7R4 showed minimal activity (0.064 nmol/mg of protein), as did the lcb1-100 (0.082 nmol/mg of protein) protein extract (Fig. 2A). Thus, protein extracted from lcb1-100 had minimal _in vitro_ serine palmitoyltransferase activity compared with the wild type protein extracts. To determine if _in vitro_ inactivity of serine palmitoyltransferase from lcb1-100 affected the steady state sphingolipid synthesis _in vivo_, we labeled both wild type and the lcb1-100 strains with tritiated palmitate for 6 h at 30 °C. Palmitate is converted to palmitoyl-CoA by the cells and then condensed with serine, by serine palmitoyltransferase, to form keto-dihydrosphingosine (Fig. 4). Keto-dihydrosphingosine is then further metabolized to the complex sphingolipids. The base-hydrolyzed organic extract of strain lcb1-100 had labeled bands corresponding to sphingoid bases and complex sphingolipids comparable to the levels seen in the RH406 extract (Fig. 2B). Bands corresponding to dihydrophosphoglycerine, phytosphingosine, inositol phosphoceramide, mannose inositol phosphoceramide, and mannose (inositol-phospho) ceramide were present in both extracts (Fig. 2B). Furthermore, high pressure liquid chromatographic analysis of Bligh and Dyer extracts of the lcb1-100 strain were found to contain normal levels of the expected four species of sphingoid bases found in yeast (data not shown). Therefore, despite minimal _in vitro_ enzyme activity, lcb1-100 was found able to produce sphingolipid levels comparable to those of the isogenic wild type at 30 °C. These results suggest that the point mutation in _LCB1_ renders the enzyme activity labile in cell extracts but normal function is maintained _in vivo_.

Heat stress has been shown previously to induce a transient increase in sphingolipid bases of wild type strains. Therefore, the increased production of sphingoid bases upon heat stress was examined in the wild type yeast strain RH406 (Fig. 2C) and heat-sensitive serine palmitoyltransferase strain lcb1-100 (Fig. 2D). Lipid extracts from RH406 showed increases in C18 phytosphingosine (252%), C20 phytosphingosine (435%), C18 dihydrosphingosine (238%), and C20 dihydrosphingosine (289%) by 15 min at 39 °C. In contrast the lcb1-100 strain lipid extracts showed no increases in any of the four measured sphingoid bases (Fig. 2D). Therefore, lcb1-100 yeast cells have normal steady-state levels of sphingolipids, but were unable to produce an increase of the sphingoid bases upon heat stress.

Next strains lcb1-100 and RH406 were evaluated for the transient cell cycle arrest by both budding and cell cycle analysis. The RH406 cells showed a large arrest at 1 h of heat stress with only 24% of the cells budded compared with 64% of cells budded in the asynchronous culture. The cell cycle analysis of RH406 showed large G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M peaks with an intervening S phase area (Fig. 3) and some post G<sub>2</sub>/M cells. The cell cycle plot showed a significant loss of S phase area at the 1 h of 39 °C time point corresponding to the loss in budding. Therefore, the transient G<sub>0</sub>/G<sub>1</sub> arrest seen at 1 h of heat stress by budding appeared as a loss of S phase area in the cell cycle analysis. In contrast to the RH406 budding loss, the percentage of budded lcb1-100 cells did not change with 1 h (67%) of heat stress as compared with asynchronous cells at 30 °C (67%). The cell cycle of lcb1-100 displayed a pre-G<sub>0</sub>/G<sub>1</sub> peak of debris and then peaks for G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M with an intervening S phase area. The asynchronous cell cycle profile of lcb1-100 was maintained (Fig. 3) at 1 h of heat stress, corresponding to the lack of change in the percentage of budded cells. These data demonstrate a necessary role for the _de novo_ induction of sphingolipids via serine palmitoyltransferase activity in the heat-induced transient G<sub>0</sub>/G<sub>1</sub> arrest.

Epistasis Analysis of Sphingolipid Mutants Suggests That Free Sphingoid Bases Are the Likely Active Species in the Cell Cycle Arrest Response to Heat Stress—The recent cloning of many of the genes involved in the sphingolipid synthesis and breakdown pathways (Fig. 4) now allows for the analysis of bioactive species by utilizing key mutants in these pathways.

Production of sphingoid base phosphates has been shown to occur mainly through Lcb4 and minimally through Lcb5 (23). Table II shows that each of these knockouts arrested and recovered upon heat stress as measured by the percentage of S phase. Moreover, the double knockout of both _LCB4_ and _LCB5_ also arrested (S phase not detectable). However, the double deletion of _LCB4_ and _LCB5_ did not recover by 2 h of heat stress as shown by no detectable cells in the S phase area. The isogenic wild type strain JK93d showed no detectable cells S phase area at 1 h of 39 °C and recovered to 34% S phase by 2 h of 39 °C. These data show that the two sphingoid base kinases are not required for the transient arrest but are required for the recovery phase.

Ceramide production has been found to be greatly decreased in the _ELO2_ deletion mutant, probably as a result of a deficiency in the very long chain fatty acids that are normally acylated at the amino group of the sphingoid bases (24). The knockout of _ELO2_ was arrested (S phase not detectable) and recovered (38%) as well as the isogenic wild type JK93d. There-
fore, ceramide production is not likely to play a role in the transient arrest induced at 39 °C. Also tested were the knockout of YSR2, a sphingoid base phosphate phosphatase (18) and the knockout of DPL1, the dihydrosphingosine phosphate lyase (25) (Table II). The knockout of YSR2 became arrested (2% S phase) and recovered (33% S phase) upon heat stress. The knockout strain of DPL1 also arrested with 4% S phase at 1 h at 39 °C. By 2 h at 39 °C, the knockout strain of DPL1 recovered to 13% of cells in S phase. These data provide further evidence that the bioactive species are not the sphingoid base phosphates.

Finally, the knockout strain of SYR2, the dihydrosphingosine hydroxylase, which catalyzes conversion of dihydrosphingosine to phytosphingosine (26), was analyzed for cell cycle upon heat stress. This strain arrested at 1 h (2% S phase) and recovered by 2 h (13% S phase) at 39 °C. Similar results were obtained using the isogenic wild type W303 (Table II). This indicates that phytosphingosine is not needed for arrest, but still could be sufficient to induce arrest (see below). Overall, the epistasis analysis indicates that sphingoid bases themselves are the best candidates for the active molecules responsible for mediating the cell cycle arrest.

Exogenous Sphingoid Bases Cause a Transient Cell Cycle Arrest—The conclusion from the epistasis analysis that the sphingoid bases are the bioactive agents in the cell cycle arrest led us to investigate the effects of exogenous sphingoid bases. Despite being found not to be necessary for the cell cycle arrest by the aforementioned genetic analysis, phytosphingosine was found to induce arrest at 10 μM or higher (Fig. 5A) by 30 min of treatment. The arrest by phytosphingosine was very rapid in that S phase was significantly decreased by 20 min to 11% compared with an asynchronous baseline of 30% and caused a nearly complete arrest (4% S phase) at 30 min (Fig. 5B). Treatment with 20 and 40 μM phytosphingosine showed a large decrease in S phase at 20 min and maintained a full arrest for up to 60 min (data not shown).
Sphingolipid mutants

Scheme shows the enzymes of sphingolipid biosynthesis and breakdown. SPT, serine palmitoyltransferase; KeDHS, keto-dihydrosphingosine; LCBKP, sphingoid base kinase; YPC1, lipid biosynthesis and breakdown. SPT, serine palmitoyltransferase; YDC1, yeast dihydroceramidase; IPC, inositol phosphate phosphatase; DHSH, dihydrosphingosine hydroxylase; and CS, ceramide synthase. Figure courtesy of Dr. Cungui Mao. Deletions used in Table II are underlined in the scheme.

Table II

Heat-induced transient cell cycle arrest analysis of sphingolipid mutants

Log phase 7R4-LCB1 cells were fixed, stained with propidium iodide, and modeled by ModFit LT. A, graph represents percentage S phase at 30 min of treatment with the given concentrations of phytosphingosine. B, graph shows the time course of arrest using 10 µM phytosphingosine.

Fig. 5. Dose and time dependence of phytosphingosine-induced arrest. Cells of the yeast strain 7R4-LCB1 were treated with phytosphingosine (pSph) as denoted and run through flow cytometry and modeled by ModFit LT. A, graph represents percentage S phase at 30 min of treatment with the given concentrations of phytosphingosine. B, graph shows the time course of arrest using 10 µM phytosphingosine.

Fig. 4. Representation of sphingolipid biosynthesis and breakdown in S. cerevisiae. Scheme shows the enzymes of sphingolipid biosynthesis and breakdown. SPT, serine palmitoyltransferase; KeDHS, keto-dihydrosphingosine; LCBK, sphingoid base kinase; YPC1, yeast dihydroceramidase; IPC, inositol phosphoceramide; MIPC, mannose inositol phosphoceramide; M(IP)2C, mannose (inositol phosphate)2 ceramide; LCBPP, sphingoid base phosphate phosphatase; DHSH, dihydrosphingosine hydroxylase; and CS, ceramide synthase. Figure courtesy of Dr. Cungui Mao. Deletions used in Table II are underlined in the scheme.

Overall, these data show significant specificity to the naturally occurring sphingoid bases.

Cln3 Acts Downstream of the Sphingolipid Bases in the Cell Cycle Arrest Pathway—Cyclins have been shown to be key players in the heat stress-induced and alpha factor-induced G1/G2 arrest (27). Furthermore, previous studies have shown that a hyperstable Cln3 (CLN3-1) is resistant to cell cycle arrest upon heat stress. Therefore, it became of interest to determine whether cyclins function downstream of the sphingoid bases. Strain LDW6A (CLN3-1) and its isogenic wild type (GR2) were tested in response to heat stress. As expected, LDW6A was found to lack the heat stress-induced cell cycle arrest seen in the wild type GR2 (7) (data not shown). The effect of treatment with 20 µM phytosphingosine on the cell cycle of strains GR2 and LDW6A was next evaluated. Strain GR2 showed a loss of the S phase area in its cell cycle profile at 30 min, with a recovered S phase area by 120 min (Fig. 6A). The Cln3-1-containing strain LDW6A did not show any loss of S phase area (Fig. 6B) over the time course of phytosphingosine treatment. Therefore, Cln3 functions downstream of the sphingoid bases to prevent their effect on inducing a transient cell cycle arrest.

Possible regulators of Cln3 were also evaluated to determine whether they are required in the heat-induced cell cycle arrest pathway. As cyclin Cln3 has been shown to be also regulated by the alpha factor/ MAPK pathway (27), the knockout strain of the MEKK (STE11) of this pathway was tested with heat stress. Heat-stressed Δste11 cells showed 1.11% of cells in S phase at 45 min. The time point of 45 min was used, because this was the optimal time when the isogenic wild type strain 4741 was arrested (Table IV). Therefore, inactivation of the alpha factor-activated MAPK pathway by STE11 knockout did not affect the ability of heat stress to arrest the cell cycle. We further evaluated other potential regulators of Cln3 by using a knockout strain of the MEKK (BCK1) of the stress-induced MAPK pathway (27). The Δbck1 cells were still able to arrest (2% S phase) at 45 min of heat stress (Table IV). Thus, inactivation of the stress-induced MAPK pathway by BCK1 knockout did not affect the heat stress-induced cell cycle arrest. These

2 G. Jenkins, unpublished observations.
after treatment with 20 μM of yeast strains GR2 and LDW6A were treated with 20 μM sphingosine (L-erythro-dihydrosphingosine (L-e dhsph), L-erythro-sphingosine (L-e sph), or stearylamine. Cells were taken at the given time, fixed, stained with propidium iodide, and run on flow cytometry. Data were analyzed by ModFit LT, and the percentages of S phase are presented.

| Treatment, 20 μM | % S phase |
|------------------|-----------|
|                  | 10 min | 20 min | 30 min | 45 min | 60 min | 120 min |
| Control          | 26.79  | 28.35  | 28.71  | 28.30  | 27.85  | 29.20   |
| psph             | 21.93  | 6.33   | 1.96   | 0.55   | 1.54   | 19.75   |
| d-e dhsph        | 18.33  | 8.69   | 2.55   | 3.17   | 6.89   | 17.52   |
| L-e dhsph        | 23.51  | 9.42   | 7.50   | 12.80  | 27.60  | 18.52   |
| d-e sph          | 23.76  | 8.77   | 3.15   | 3.06   | 2.40   | 10.34   |
| Stearylamine     | 25.36  | 19.92  | 21.98  | 36.65  | 25.48  | 27.95   |

TABLE III
Specificity of sphingoid bases in causing a transient cell cycle arrest

Log phase yeast cells of strain TR4-LCB1 were treated with 20 μM of phytosphingosine (psph), L-erythro-dihydrosphingosine (L-e dhsph), L-erythro-sphingosine (L-e sph), or stearylamine. Cells were taken at the given time, fixed, stained with propidium iodide, and run on flow cytometry. Data were analyzed by ModFit LT, and the percentages of S phase are presented.

Fig. 6. Cell cycle analysis of strains GR2 and LDW6A (CLN3-1) after treatment with 20 μM phytosphingosine. Log phase cultures of yeast strains GR2 and LDW6A were treated with 20 μM phytosphingosine (psph), and the resultant cell cycle was determined by flow cytometry at the given time points.

data show that neither the alpha factor or stress-induced MAPK pathways are required for heat stress-induced cell cycle arrest.

Cyclin Cln3 has also been shown to be ubiquitinated and subsequently degraded (28), suggesting a possible mechanism of regulation. Also, it was previously shown that de novo synthesis of sphingolipids regulates a ubiquitin pathway through Doa1 (15). Therefore, the knockdown strain of a regulatory component of the proteasome pathway, DOA1, was evaluated and found to arrest (Table IV) at 45 min of heat stress with 1% of the cells in S phase. These data indicate that the ubiquitination and degradation pathway is not required for the heat-induced cell cycle arrest.

The Sphingoid Base Kinases Likely Mediate Cell Cycle Recovery by Sphingoid Base Clearance—The aforementioned epistasis analysis led to the finding that the double-knockout of the sphingoid base kinases was deficient in the recovery from the heat-induced cell cycle. Therefore, the Δlcb4,5 strain was tested over a longer time course of heat stress (Fig. 7A). The Δlcb4,5 strain showed an arrest at 1 h with the expected large decrease in the S phase area. By 2 h, the G1/M phase had increased to a large broad-shouldered peak (77% of cells). The 3- and 4-h heat stress time points confirmed this finding, because the majority of cells were found in the G1/M peak (87 and 79%, respectively). However, by 4 h of heat stress the double-knockout strain had some S phase (3%) and an increased G1/G0 peak compared with the 3-h time point (7% compared with 18%) indicating some recovery of a normal cell cycle. These data indicate two possibilities for the sphingoid base kinases to play. One possibility is that the sphingoid base phosphates play a role in the recovery of the normal cell cycle upon heat stress.

The other possibility is that cleavage of the sphingoid base via the kinase pathway removes the arrest signal and thus allows cell cycle recovery.

The sphingoid base kinases have been shown to act on phytosphingosine, sphingosine, and dihydrosphingosine (23). However, the yeast biosynthetic pathway through ceramide synthase appears to be able to incorporate only the endogenous sphingoid bases, phytosphingosine and dihydrosphingosine, and not sphingosine. Therefore, sphingosine appears to go through the kinases for clearance and/or conversion to other products. Therefore, we first tested the effect of phytosphingosine on the kinase double-knockout strain. Treatment of the Δlcb4,5 strain with 20 μM phytosphingosine resulted in a loss of S phase area by 1 h (Fig. 7B). The S phase area of the double-knockout strain was partially recovered by the 2-h treatment and completely recovered by the 4-h time point. Ergo, recovery was not dependent upon the making of phosphorylated phytosphingosine through the sphingoid base kinases. Next, the kinase double knockout was treated with 20 μM sphingosine. Sphingosine treatment also caused the expected cell cycle arrest by 1 h; however, the arrest was not reversed over the 4 h of treatment tested (Fig. 7C). The 4-h time point of 20 μM sphingosine had no detectable S phase. Therefore, the inability of the double knockout of the sphingoid base kinases to either metabolize sphingosine in the yeast sphingolipid biosynthetic pathway or through phosphorylation by the kinases led to a prolonged arrest of its cell cycle. Taken together these results suggest that the clearance hypothesis of alleviating the cell cycle arrest is the more likely explanation.

DISCUSSION

The need for sphingolipids in the heat stress response has been established with several studies, which show that sphingolipids provide a distinct advantage in growth of yeast at higher temperatures (9–11, 15). The questions now being addressed in current research are 3-fold. First, the source of the sphingolipids generated in response to heat stress are being studied as well as the mechanisms involved in regulating sphingolipid metabolism. Second, the various sphingolipids generated are being examined for possible roles as bioactive molecules. Finally, the mechanisms by which sphingolipids mediate their roles in the heat stress response are being evaluated. Recent research has shown large increases in the sphingolipid bases and, subsequently, the ceramides upon heat stress (9, 11). The primary source of the increased levels of sphingolipid bases has been shown to be de novo synthesis (9, 11). The data presented in this study and other studies are beginning to define key roles for the de novo production of sphingolipid bases through serine palmitoyltransferase.

The current study defines a vital role for de novo production of sphingolipids as needed for the transient cell cycle arrest, which occurs upon an upshift in temperature from 30 to 39 °C. Using the strain with a suppressor of the LCB1 knockout, TR4,
Sphingoid Base Biosynthesis in Cell Cycle Arrest of S. cerevisiae

Log phase yeast cells were treated for the indicated times at 39 °C and compared with asynchronous (ASY) cells. Cells were fixed, stained, and run on flow cytometry. Data were analyzed by ModFit LT, and the percentages of S phase are presented. Strains used were the isogenic wild type 4741, knockout of the alpha factor MAPK pathway MEKK (STE11), knockout of the stress MAPK pathway MEKK (BCK1), and the knockout of the regulatory component of the proteasome pathway (DOA1).

Table IV
Analysis of possible regulators of Cln3 via mutants

| Strain   | ASY 60 min 39 °C | 30 min 39 °C | 45 min 39 °C | 60 min 39 °C | 120 min 39 °C |
|----------|------------------|-------------|-------------|-------------|----------------|
| 4741     | 28.37            | 32.79       | 28.78       | 24.70       |                |
| Δste11   | 7.80             | 7.41        | 9.11        | 5.48        |                |
| Δbck1    |                 |             |             |             |                |
| Δdoa1    |                 |             |             |             |                |

![Graph](image)

**FIG. 7.** Cell cycle of strain Δlcba, Icb5 after heat stress and treatment with sphingosine and phytosphingosine. Log phase cultures of strain Δlcba, Icb5 were treated as denoted, and the resultant cell cycle was determined by flow cytometry at the given time points. A, cells were upshifted from 30 to 39 °C. B, cells were treated with 20 μM phytosphingosine (psph). C, cells were treated with 20 μM sphingosine (sph). Graphs were generated by ModFit LT.

we showed that sphingolipids are needed for the aforementioned arrest. Strain lcba100 was characterized and found to make the normal complement of sphingolipids at 30 °C but to lack the increased de novo synthesis of sphingoid bases upon heat stress. Importantly, this lcba100 strain was found to be deficient in the transient cell cycle arrest seen after heat stress. Therefore, the immediate conclusion from this study is that this cell cycle arrest is dependent upon the de novo production of sphingolipids.

To address the question of which sphingolipid(s) is (are) a candidate for regulating this process, we performed an analysis of the key mutants of the sphingolipid biosynthetic and breakdown pathways in terms of the heat-induced transient cell cycle arrest. These data showed that the active species was most likely the free sphingoid bases. This finding was furthered by studies showing that exogenous treatment with sphingoid bases was able to induce the transient arrest in the cell cycle, thus reproducing the effects of heat stress.

The transient G1/G0 arrest caused by heat stress has been shown to be blocked by the hyperstable Cln3-1 (7). In this study, it was found that a strain containing the hyperstable Cln3 was found to not arrest upon treatment with 20 μM phytosphingosine. Therefore, Cln3 acts downstream of sphingoid bases in the pathway leading to cell cycle arrest. To try and further define these pathways, we tested three likely candidates. Two MAPK pathways thought or known to regulate Cln3 are the alpha factor pathway and stress-induced pathway (27). However, in the case of heat stress-induced cell cycle arrest, the two MAPK pathways tested were found to not be involved.

Next, the ubiquitin pathway was tested because Cln3 has been shown to be phosphorylated, then ubiquitinated, and finally degraded (28). This form of regulation was further suggested by the recent finding of sphingolipid regulation of ubiquitin interpretation and proteolysis of nutrient permeases (15). However, regulation by this mechanism was shown to not be involved in the cell cycle arrest. Thus, our research opens the possibility of a novel mechanism of regulation by sphingoid bases of Cln3-mediated cell cycle arrest.

On the other hand, recovery from the heat-induced cell cycle arrest was found to be mediated through the sphingoid base kinases (LCB4, LCB5). We considered two possible roles for the kinases in this effect. Either the sphingoid base phosphates, the products of the sphingoid base kinases, are needed for recovery of an asynchronous cell cycle, or the kinases are required for the clearance of the sphingoid bases to remove the signal of arrest. We showed that clearance is the more likely mechanism, because the double knockout of the sphingoid base kinases recovered from phytosphingosine-induced arrest but not from sphingosine arrest; the latter required specifically the action of the kinases for metabolic clearance. Also, the knockout of DPL1, the dihydrophosphingosine phosphate lyase, was shown to have a slowed recovery of S phase (14%) compared with its isogenic wild type (34%) (Table II). These data are also consistent with this mechanism, because the loss of the ability to clear sphingoid base phosphates affects the overall clearance of the sphingoid bases, thus affecting recovery from arrest. Clearly, the roles of kinases and the lyase are important and need further study in the heat stress response.

The study of sphingolipids in the yeast heat stress response has rapidly progressed. Now, one can explore the roles of the various sphingolipids, especially sphingoid bases, because they have been implicated in the trehalose response, possibly gene regulation through stress response elements (10), ubiquitination pathway (15), and now the transient cell cycle arrest response. Furthermore, yeast sphingoid bases have been implicated in the regulation of the internalization step of endocytosis likely through protein phosphorylation (29, 30). Also, the study of the regulation of the enzymes involved in the production of the sphingolipids will prove to be exciting, especially the regulation of serine palmitoyltransferase upon heat stress and possibly other responses. Finally, how and through what pathways the sphingolipids mediate responses to various stimuli.
can now be studied. Overall, sphingolipids are emerging as key molecules in the yeast heat stress response.

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