Sterol-dependent Regulation of Sphingolipid Metabolism in
Saccharomyces cerevisiae*

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We had previously isolated the temperature-sensitive erg26-1 mutant and characterized the sterol defects in erg26-1 cells, examined their effects on cell growth, and initiated studies designed to elucidate how might changes in sterol levels coordinately regulate sphingolipid metabolism in Saccharomyces cerevisiae. Using [3H]inositol radiolabeling studies, we found that the biosynthetic rate and steady-state levels of specific hydroxylated forms of inositolphosphorylceramides were decreased in erg26-1 cells when compared with wild type cells. [3H]Dihydrorhosphosine radiolabeling studies demonstrated that erg26-1 cells had decreased levels of the phyto- sphingosine-derived ceramides that are the direct pre- cursors of the specific hydroxylated inositol phospho- rylceramides found to be lower in these cells. Gene dosage experiments using the sphingolipid long chain sphingoid base (LCB) hydroxylase gene, SUR2, suggest that erg26-1 cells may accumulate LCB, thus placing one point of sterol regulation of sphingolipid synthesis pos- sibly at the level of ceramide metabolism. The results from additional genetic studies using the sphingolipid hydroxylase and copper transporter genes, SCS7 and CCC2, respectively, suggest a second possible point of sterol regulation at the level of complex sphingolipid hydroxylation. In addition, [3H]inositol radiolabeling of sterol biosynthesis inhibitor-treated wild type cells and late sterol pathway mutants showed that additional blocks in sterol biosynthesis have profound effects on sphingolipid metabolism, particularly sphingolipid hydroxylation state. Finally, our genetic studies in erg26-1 cells using the LCB phosphate phosphatase gene, LBP1, suggest that increasing the levels of the LCB sphingoid base phosphate can remediate the temperature-sensitive phenotype of erg26-1 cells.

Sphingolipids are ubiquitous membrane lipids that are found in all eukaryotic cells. They are structural components of lipid bilayers (1), have emerged as signaling molecules generated in response to a variety of growth modulators (2–5), and have been found to play an important role in regulating vesicular and lipid trafficking events in higher eukaryotes (6–8). With regards to human pathology, a number of diseases have been attributed to the inappropriate trafficking and/or metabolism of sphingolipids. Human Niemann-Pick disorders are charac- terized by the intercellular mislocalization and accumulation of sphingolipid and cholesterol in lysosomes (9–11), and a number of rather severe sphingolipid storage diseases result from the inability of a diseased cell to properly catabolize sphingolipids (12, 13).

The mechanisms regulating sphingolipid metabolism at the molecular level are not that well understood. However, there is some evidence suggesting that sphingolipid biosynthesis may be regulated coordinately with cholesterol metabolism in higher eukaryotes. Storey et al. (14) showed that sphingomyel- in and ceramide biosynthesis could be blocked by the addition of the HMG-CoA reductase inhibitor lovastatin. They also dem- onstrated that a cholesterol auxotrophic Chinese hamster ovary cell line harbor defects in SREBP1 processing and cholesterol biosynthesis had greatly decreased levels of sphin- gomyelins (14). However, others showed that lovastatin treat- ment of CaCo-2 cells did not result in changes in sphingolipid biosynthesis (15). There is work from several laboratories showing that sterol esterification rapidly occurs in response to membrane depletion of sphingomyelin (16–18) and is accom- panied by down-regulation of de novo sterol biosynthesis brought about by sterol-dependent inhibition of SREBP process- ing (19). Recently, Worgall et al. (20) showed that increasing ceramide levels or inhibiting ceramidase activity in Chinese hamster ovary cells decreases SRE-mediated transcription, presumably by reducing the levels of the transcriptionally ac- tive forms of SREBP-1 and SREBP-2. Thus, ceramide may mediate sphingolipid-dependent regulation of sterol metabol- ism in animals.

Genetic studies in the yeast Saccharomyces cerevisiae have hinted at the possibility of a similar coordinate regulatory system in lower eukaryotes. Genome expression studies using antifungals targeting sterol biosynthesis have revealed that many lipid metabolic genes are transcriptionally regulated in response to changes in sterol levels (21, 22). Among those genes regulated were the SUR2 LCB hydroxylase gene required for the production of phytosphingosine and the LCB1 serine palmi-

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Strain Construction and Plasmids—Yeast null mutants were generated by the one-step disruption method of Rothstein (34) using individual YIp deletion constructs (35) or the KanMX cassette (36). Yeast strains harboring individual deletions were verified by PCR analysis. The LEU2- and URA3-containing 2μ plasmids, pRS425 and pRS426, respectively, were used to construct the various high copy vectors used in this study (35). The glycerol-3-phosphate dehydrogenase promoter (GPD) (37) was obtained from the vector pJR1130 that was provided by Drs. Jasper Rine and Chris Beh (University of California, Berkeley, CA). The GPD promoter was subcloned into pRS425 and pRS426, and these vectors were used for the overexpression studies. A 2μ vector overexpressing CCC2 was kindly provided by Dr. Valeria Culotta (Johns Hopkins University School of Public Health, Baltimore, MD). CCC2 was excised from this vector and subcloned into pRS426 that contained the GPD promoter. All of the genomic sequences subcloned into the various vectors were obtained by PCR amplification using the high fidelity Pfu polymerase. All of the DNA sequences that were generated by PCR were sequenced and compared with the yeast genome database.

Metabolic Radiolabeling and Analysis of Lipids—Starting cultures for all of the lipid radiolabeling experiments were grown overnight at 27 °C to exponential phase in synthetic complete media or selective minimal media. A₀₀ were then taken, and the cultures were diluted to 5 × 10⁶ cells/ml and preincubated at 27 and 37 °C for 3 h before antifungal treatment and/or radiolabeling. Radiolabeled lipids were resolved by TLC and visualized by autoradiography (Kodak XAR5). The percentage value of each lipid species was determined by densitometry using Bio-Rad model GS-670 Imaging Densitometer and Molecular Analyst software, version 1.4.1.

For pulse radiolabeling of complex sphingolipids, the cultures were incubated with 5 μCi/ml [3H]myo-inositol for 30 min. For steady-state labeling, the cultures were incubated with 1 μCi/ml [3H]myo-inositol for 5 h. Radiolabeled sphingolipids were extracted with ethanol, water, diethyl ether, water, diethyl ether, pyridine, 4.2 N ammonium hydroxide (15:15:5:10:0.018), treated with methylamine as described (38), and analyzed by one-dimensional TLC using chloroform, methanol, acetate acid, and water (16:6:4:1.6). When necessary, wild type cultures were preincubated with specified antifungal compounds as described for the analysis of sterols (see above).

For pulse radiolabeling of ceramides, cultures were incubated with 5 μCi/ml [3H]dihydrosphingosine for 20 min. For steady-state radiolabeling, the cultures were incubated with 5 μCi/ml [3H]dihydrosphingosine for 30 min, pelleted, and washed in media lacking radiolabel, and chased for an additional 3 h. To help resolve the various ceramides species, wild type cultures were incubated in the absence and presence of 140 μM fumonisin B1. Radiolabeled ceramides were extracted with ethanol, water, diethyl ether, pyridine, 4.2 N ammonium hydroxide.
RESULTS

The erg26-1 Mutant Harbors Defects in Complex Sphingolipid Metabolism—We first analyzed the complex sphingolipid compositions of wild type and erg26-1 cells using lipid radiolabeling studies and TLC. In these studies, we examined the rates of biosynthesis and steady-state levels of sphingolipids. Our experiments were carried out at both the permissive and restrictive temperatures for erg26-1 cell growth. The results of these studies are shown in Fig. 2.

Using [H]inositol radiolabeling studies, we found that erg26-1 cells harbored defects in the rate of biosynthesis of several hydroxylated IPC species (Fig. 2). These defects were detected at both temperatures in erg26-1 cells. Using yeast mutants lacking individual sphingolipid hydroxylase activities (23, 24, 42), we were able to determine what IPC species were affected by the erg26-1 mutation. Our results revealed that erg26-1 cells grown at the permissive temperature had an increased rate of biosynthesis of IPC-C (4.2-fold) while exhibiting a decreased rate of biosynthesis of IPC-D (8.5-fold) when compared with wild type cells grown under the exact conditions described previously (38).
We found that erg26-1 cells were defective in their ability to synthesize and accumulate the phytosphingosine-containing ceramides, ceramide-B and ceramide-C (Fig. 3). Ceramide-B is the precursor for ceramide-C, where ceramide-C is produced through the action of the sphingolipid hydrolase, Scs7p (Fig. 1) (23). The rate of biosynthesis and steady-state levels of ceramide-B and -C were most severely reduced at the permissive growth temperature (Fig. 3, B and D). The steady-state levels of ceramide-B and -C in temperature-shifted erg26-1 cells were only 22 and 28% of that seen in wild type cells (Fig. 3D). The rate of biosynthesis and steady-state levels of the dihydrophosphingosine-containing ceramides, ceramide-A and ceramide-B', in erg26-1 cells were similar to those seen in wild type cells (Fig. 3). We were unable to resolve ceramide-D using this TLC method. Therefore, we cannot say whether the biosynthetic and/or the metabolic level of this ceramide species was affected by the erg26-1 mutation.

### erg26-1 Cells Have Defects in Fatty Acid Biosynthesis

C26 fatty acids are used exclusively to synthesize complex sphingolipids in yeast (43). We reasoned that the defects seen in complex sphingolipid metabolism in erg26-1 cells may cause the accumulation of C26 fatty acids, which in turn may have secondary effects on total fatty acid metabolism. Thus, we determined the steady-state levels of various fatty acid species in wild type and erg26-1 cells at the permissive and restrictive temperatures using GC/MS analysis.

We found that at either temperature, erg26-1 cells contained dramatically less C10:0 and C12:0 fatty acid species while overaccumulating C26:0 fatty acid (Fig. 4A). erg26-1 cells had 4- and 3-fold reductions in C10:0 and C12:0 fatty acids while accumulating ~3-fold higher C26:0 fatty acid at both growth temperatures. In addition, we found that erg26-1 cells also accumulated low levels of C20:0, C20:1, and C24:0 fatty acid species at the restrictive temperature (~0.03–0.10% of total fatty acid) (not shown). In contrast, the levels of the major fatty acid species in yeast cells, C16:0, C16:1, C18:0, and C18:1, were similar in erg26-1 and wild type cells at both temperatures (Fig. 4B).

**Figure 3.** erg26-1 cells have lower levels of phytosphingosine-derived ceramides. The rates of biosynthesis (A and B) and steady-state levels (C and D) of dihydro- and phytosphingosine-derived ceramides were determined by 3H-dihydrosphingosine radiolabeling of wild type (black bars) and erg26-1 (white bars) cells as described under “Experimental Procedures.” The experiments were performed at the permissive (A and C) and restrictive (B and D) growth temperatures. cer-A, ceramide-A; cer-B', ceramide-B'; cer-B, ceramide-B; cer-C, ceramide-C. The LCB backbone of ceramide-A is dihydrosphingosine. Ceramide-B' is derived from ceramide-A and contains an additional hydroxyl group attached to the fatty acid moiety. The LCB of ceramide-B is phytosphingosine. Ceramide-C is derived from ceramide-B and contains an additional hydroxyl group attached to the fatty acid moiety.

### Altering Long Chain Base Levels in erg26-1 Cells Affects Cell Viability

The loss of proper ceramide-B levels in erg26-1 cells could be due either to a decrease in its biosynthesis or an increase in its turnover or both. This may lead to the accumulation of cytotoxic levels of LCB and/or LCBP at high temperatures (44–47). To begin to examine whether LCB and/or LCBP may accumulate in erg26-1 cells and contribute to the observed growth defect, we took a genetic approach and first determined how altering the gene dosage of individual hydroxylase genes affected erg26-1 viability. In particular, we were interested in determining how the increased gene dosage of the SUR2 gene required for the biosynthesis of the LCB phytosphingosine affected erg26-1 viability (23, 24).

Wild type and erg26-1 cells carrying individual high copy plasmids overexpressing SUR2, SCS7, or CCC2 from the constitutive GPD promoter were examined for their ability to grow at the permissive and restrictive growth temperatures. SUR2 and SCS7 encode for sphingolipid hydrolases (23, 24), whereas CCC2 encodes for a copper transporter that is required for Cu2+ uptake (Fig. 1) (42). Beeler et al. (48) have shown that the synthesis of fully hydroxylated IPC-D is dependent on CCC2 and the concentration of Cu2+ in the media.

As shown in Fig. 5, we found that the overexpression of each gene individually was not toxic to wild type cells grown at either temperature (Fig. 5, W303-1A, A and C). In contrast, the constitutive overexpression of either SUR2 or CCC2 alone in
erg26-1 cells was highly toxic to cell growth at the permissive temperature of 27 °C (Fig. 5, erg26-1, B), whereas the overexpression of SCS7 had no effect on cell growth at the permissive temperature and actually suppressed the temperature sensitive (ts) defect of erg26-1 cells (Fig. 5, erg26-1, B and C).

Based on these results, we predicted that the constitutive overexpression of SUR2 increased LCB and/or LCBP levels in erg26-1 cells, because these cells are defective in ceramide-B metabolism. This suggests that accumulation of one or both of these LCBs may be responsible for the SUR2-dependent cell toxicity observed at low temperatures. To determine whether the increased accumulation of LCB or LCBP contributes to the observed SUR2-dependent growth defect of erg26-1 cells, we constructed erg26-1 strains lacking either the LBP1/YSR2/LCB3 or LBP2/YSR3 phosphatase gene (46, 49, 50) and determined their growth viability when SUR2 was overexpressed.

Using these strains, we found that the loss of LBP1, but not LBP2, remediated the SUR2-dependent cell toxicity of erg26-1 cells at low temperatures (Fig. 6, A–C). In fact, the loss of LBP1 suppressed the ts phenotype of erg26-1 cells (Fig. 6D). Interestingly, the loss of LBP2 in erg26-1 cells resulted in drastically less growth at low temperatures, similar to that seen in erg26-1 cells overexpressing SUR2 (Fig. 6, A versus C, erg26-1 2μ versus erg26-1 lbp2 2μ). lbp1-dependent suppression of SUR2-dependent toxicity was not acting through decreasing the accumulation of toxic zymosterol intermediates, because these lipids accumulated to similar levels in all strains (Fig. 6E).

Together our genetic results point to the accumulation of LCB contributing to SUR2-dependent cell toxicity rather than the accumulation of LCBP. In fact, we suggest that increased accumulation of LCBP can actually remediate the cell toxicity associated with increased accumulation of zymosterol intermediates at high temperature. These hypotheses are substantiated by the fact that we were unable to generate erg26-1 strains lacking the LCB4 gene encoding the major LCB kinase in yeast (51). Presumably, this strain would accumulate very high levels of LCB. We could generate erg26-1 strains lacking the LCB5 gene encoding a second minor LCB kinase (51). erg26-1 lcb5 cells showed similar growth characteristics to erg26-1 strains overexpressing SUR2 when SUR2 was overexpressed in this double mutant (not shown). Finally, we found that overexpressing either the LAG1 or LAC1 putative ceramide synthase genes in erg26-1 cells remediated SUR2-dependent cell toxicity at low temperatures and allowed for weak suppression of the ts phenotype (not shown).

Specific Hydroxylated Sphingolipids Play Roles in SCS7 and CCC2 Effects on erg26-1 Viability—Scs7p hydroxylates both dihydro- and phytosphingosine-derived IPC species, whereas Ccc2p is required only for the hydroxylation of the phytosphingosine-derived IPC-C and subsequent production of IPC-D (Fig. 1). To begin to (i) define which hydroxylated sphingolipid species accumulates and is required for SCS7-dependent ts suppression and (ii) further substantiate that the CCC2-dependent cell toxicity results from increased accumulation of fully hydroxylated IPC-D, we examined how deleting SUR2 affected erg26-1 cells overexpressing SCS7 or CCC2.

Interestingly, we found that altering the gene dosage of SUR2, and thus the production of phytosphingosine-derived IPCs, had no effect on SCS7-dependent ts suppression, because erg26-1 sur2 cells overexpressing SCS7 remained viable at high temperature (Fig. 7, A versus B). On the other hand, the loss of SUR2 in erg26-1 cells overexpressing CCC2 completely remediated the overexpression toxicity that we previously observed at low temperature (Fig. 8A, erg26-1 2μ–CCC2 versus erg26-1 sur2 2μ–CCC2).

To further substantiate that CCC2-dependent toxicity was most likely due to the accumulation of IPC-D rather than a general cell-associated toxicity derived from increased sensitivity to divalent cations, we determined the growth of erg26-1 cells in the absence and presence of Cu2+, Mn2+, Zn2+, and Co2+. erg26-1 cells were sensitive only to the addition of Cu2+ in the media while demonstrating wild type-like sensitivity to Mn2+, Zn2+, and Co2+ supplementation (Fig. 8, B versus C–E). The increased Cu2+ sensitivity could be remediated by the addition of the copper-chelating agent bathocuproine (not shown).
Cells Harboring Specific Blocks in Sterol Biosynthesis Harbor Sphingolipid Defects—If yeast do possess mechanisms that coordinately regulate sterol and sphingolipid metabolism, then blocking sterol biosynthesis at other points in pathway should cause changes in sphingolipid metabolism. To determine whether an early block in the sterol pathway can lead to changes in sphingolipid levels, we determined the rate of biosynthesis and turnover of sphingolipids in wild type cells that were fed the Erg1p squalene epoxidase inhibitor, terbinafine, or the Erg11p C-14 demethylase inhibitor, fluconazole (43).

Using [H3]inositol radiolabeling, we found that the addition of either terbinafine or fluconazole caused a slight increase in the biosynthesis of IPC-B (1.5-fold over wild type) (Fig. 9, Pulse), which led to the sustained increased accumulation of steady-state levels of IPC-B in drug-treated cells (Fig. 9, Steady-State). In addition, we found that there was a reduction in the biosynthetic rate of production of these sphingolipids was reduced 3.5-fold. This was not due to a decrease in the levels of the IPC precursor phosphatidylinositol, because drug-treated cells had phosphatidylinositol levels that were comparable with wild type cells (not shown). IPC-D biosynthesis was not affected by drug treatment. Moreover, we found that accumulating levels of MIPC remained lower in drug-treated cells (Fig. 9, Steady-State).

Later blocks in sterol biosynthesis also perturbed sphingolipid metabolism, as evidenced by the sphingolipid defects detected in erg26-1 cells (Fig. 9, Pulse). The biosynthetic rate of production of these sphingolipids was reduced 3.5-fold. This was not due to a decrease in the levels of the IPC precursor phosphatidylinositol, because drug-treated cells had phosphatidylinositol levels that were comparable with wild type cells (not shown). IPC-D biosynthesis was not affected by drug treatment. Moreover, we found that accumulating levels of MIPC remained lower in drug-treated cells when compared with wild type cells (Fig. 9, Steady-State). These cells had lower sustained levels of IPC-D (1.7-fold lower than wild type) (Fig. 9, Steady-State).

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**DISCUSSION**

Seminal studies from several different laboratories have eloquently defined the mechanisms regulating phospholipid metabolism in the yeast *S. cerevisiae* (43, 53–55). However, the regulation of sphingolipid metabolism in this organism is far from being fully understood. The ability of the yeast to respond to changes in the environment is critical for its survival and adaptation. The mechanisms that coordinate the regulation of sterol and sphingolipid metabolism are likely to play a significant role in this process. Further research is needed to elucidate these mechanisms and their role in the regulation of sphingolipid metabolism in yeast.
less well understood. We have presented evidence that yeast may contain mechanisms that are designed to regulate sphingolipid levels in response to changes in sterols. The protein product of the ERG26 gene, 4a-carboxyethylsterol-C3 dehydrogenase, is one of three enzyme activities required for proper cyto-sterol biosynthesis (56–58). Our work has shown that loss of proper Erg26p activity leads to changes in sphingolipid levels, with sterol-dependent regulation most likely occurring at the points of ceramide biosynthesis and IPC hydroxylation. Moreover, we have shown that sterol-dependent regulation of sphingolipid metabolism in yeast seems to respond to changes in the levels of multiple sterol intermediates or possibly flux through the sterol pathway, because blocking sterol production at multiple steps in the biosynthetic pathway results in changes in particular sphingolipid levels.

One point of regulation by sterols seems to be at the level of ceramide biosynthesis. The LAG1 and LAC1 genes encode for putative ceramide synthase (59, 60). In addition, the YPC1 (61) and YDC1 (62) ceramidase genes encode proteins that hydrolyze ceramide while also being shown to catalyze in vitro ceramide production. Thus, yeast cells contain several proteins able to affect in vivo ceramide levels. Our data showed that the production of phytosphingosine-derived ceramide-B was preferentially regulated in erg26-1 cells, and dihydrophosphinosine-derived ceramide-A metabolism was far less affected (Fig. 3). This brings up the question of how sterol levels might preferentially affect the production of one ceramide species over the other. The most straightforward explanation is that regulation occurs at the level of transcription, and our laboratory is presently performing microarray studies on erg26-1 cells. Another possibility is that the level of direct sterol enzyme interaction and subsequent regulation of ceramide synthase activity. Lipids regulate many yeast phospholipid metabolic enzymes (53, 54). This type of lipid regulation would, however, have to be specific for a particular form of ceramide synthase. Interestingly, we found that when we added fumonisin to yeast cells ceramide-B production was inhibited while ceramide-A accumulated (not shown). Thus, the enzyme(s) catalyzing the production of these different ceramides shows differential specificity concerning fumonisin sensitivity.

csg2 mutants that are defective in MIPC biosynthesis are sensitive to calcium (63). Dunn et al. (64) have shown that changing the sphingolipid hydroxylation-state in csg2 cells through the loss of SCS7 remediates calcium sensitivity. Additional studies have shown that the loss of SUR2/SYR2 is associated with resistance to certain anti-fungals (24). How altering the hydroxylation-state of sphingolipids causes or is responsible for the regulation of calcium homeostasis or anti-fungal sensitivity remains to be elucidated. However, these studies suggest that properly regulating sphingolipid hydroxylation is a crucial parameter for maintaining yeast growth under multiple stress conditions.

Genes regulating yeast sphingolipid hydroxylation state include SCS7, SUR2, CCC2, and SUR7 and the two open reading frames YDL222 and YNE194 (65). Sur7p, Yd1222p, and Yne194p localize to a novel cortical patch structure, and at least Sur7p may directly interact with the cell wall (65). The sphingolipids of cells lacking either Sur7p or Ydl222p show changes in LCB length, and these cells accumulate IPC-B while containing less IPC-C and IPC-D. erg26-1 cells contain less IPC-C and IPC-D (Fig. 2). We did not determine LCB chain length composition in erg26-1 cells. Therefore, we cannot say whether erg26-1 cells completely mimic sur7 or ydl222 cells concerning total sphingolipid composition. Our preliminary studies have shown that erg26-1 cells contain very few Sur7p-containing cortical patches at low temperatures and are totally lacking these patches at high temperature. Thus, sterol-dependent regulation of sphingolipid hydroxylation may occur at the level of Sur7p-containing cortical patch biogenesis and/or morphology. We are presently examining the localization of Sur2p and Sce7p in erg26-1 cells.

LCB and LCBP affect processes in yeast including calcium signaling (66), endocytosis (67), heat shock response (45, 46, 68), and cell growth (44). We showed that altering the expression levels of genes regulating LCB and LCBP levels had profound effects on erg26-1 cell viability. Our genetic studies suggested that lowering the level of LCB or increasing the level of LCBP increased erg26-1 cell growth under certain conditions. How might an increase in LCBP levels help erg26-1 cells to grow under stress conditions? One possibility is that accumulation of LCBP in erg26-1 cells activates a compensatory Ca2+-dependent pathway. Birchwood et al. (66) recently showed that yeast mutants lacking LBP1/YSR2/LCB3 and the LCBP lyase gene DPL1 exhibited constitutively high Ca2+-accumulation and signaling. In the absence of LBP1, erg26-1 lbp1 cells, because erg26-1 cells possibly accumulate LCB, may accumulate a level of LCBP that is similar to that seen in lbp1 dpl1 cells. Ca2+-supplementation has been shown to suppress the essential requirement for ERG24 C-14 sterol reductase function (41). Moreover, studies have shown that recessive mutations that suppress the essential requirement for ERG24 also suppress the calcium sensitivity of csg2 mutants (28, 32). These results in combination with our findings would suggest that sterols directly or indirectly regulate calcium homeostasis in some way.

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