Sterol-dependent Regulation of Sphingolipid Metabolism in
Saccharomyces cerevisiae

Running Title: Regulation of yeast sphingolipids

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ABSTRACT

We had previously isolated the temperature-sensitive \textit{erg26-1} mutant and characterized the sterol defects in \textit{erg26-1} cells (Baudry, K., Swain, E., Rahier, A., Germann, M., Batta, A., Henry, K., Rondet, S., Tint, G.S., Edlind, T., Mandala, S., Kurtz, M., and Nickels, Jr., J.T. (2001) J. Biol. Chem. 276, 12702-12711). We have now determined the defects in sphingolipid metabolism in \textit{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 \textit{S. cerevisiae}. Using $[^3]H$inositol radiolabeling studies, we found that the biosynthetic rate and steady state levels of specific hydroxylated forms of inositolphosphorylceramides were decreased in \textit{erg26-1} cells when compared to wild type cells. $[^3]H$dihydrosphingosine radiolabeling studies demonstrated that \textit{erg26-1} cells had decreased levels of the phytosphingosine-derived ceramides that are the direct precursors of the specific hydroxylated inositol phosphorylceramides found to be lower in these cells. Gene dosage experiments using the sphingolipid long chain base (LCB) hydroxylase gene, \textit{SUR2}, suggest that \textit{erg26-1} cells may accumulate LCB, thus placing one point of sterol regulation of sphingolipid synthesis possibly at the level of ceramide metabolism. The results from additional genetic studies using the sphingolipid hydroxylase and copper transporter genes, \textit{SCS7} and \textit{CCC2}, respectively, suggest a second possible point of sterol regulation at the level of complex sphingolipid hydroxylation. In addition, $[^3]H$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 \textit{erg26-1} cells using the LCBP phosphatase gene, \textit{LBP1}, suggest that increasing the levels of LCBP can remediate the temperature-sensitive phenotype of \textit{erg26-1} cells.
INTRODUCTION

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 characterized by the intercellular mislocalization and accumulation of sphingolipid and cholesterol in lysosomes (9-11). While 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 that suggests sphingolipid biosynthesis may be regulated coordinately with cholesterol metabolism in higher eukaryotes. Storey et al., (14) showed that sphingomyelin and ceramide biosynthesis could be blocked by the addition of the HMG-CoA reductase inhibitor lovastatin. They also demonstrated that a cholesterol auxotrophic CHO cell line harboring defects in SREBP\(^1\) processing and cholesterol biosynthesis had greatly decreased levels of sphingomyelin (14). However, others showed that lovastatin treatment 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 accompanied by down regulation of de novo sterol biosynthesis brought about by sterol-dependent inhibition of SREBP processing (19). Recently, Worgall et al., (20) showed that increasing ceramide levels or inhibiting ceramidase activity in CHO cells decreases SRE-mediated transcription,
presumably by reducing the levels of the transcriptionally active forms of SREBP-1 and SREBP-2. Thus, ceramide may mediate sphingolipid-dependent regulation of sterol metabolism in animals.

Genetic studies in the yeast *S. 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 palmitoyltransferase gene involved in the first step of sphingolipid biosynthesis (23,24). Vik and Rine (25) have defined an SRE that is common to many *ERG* genes and is found in the promoter sequence of *LCB1* (25). And several studies examining antifungal resistance in yeast have demonstrated a genetic interaction between sterol and sphingolipid metabolism and cell viability (26-28).

*S. cerevisiae* should be an excellent model in which to study sphingolipid metabolism and regulation. Sphingolipids are essential for yeast cell viability, emphasizing their critical role in growth (29). Almost all of the genes that are required for sphingolipid biosynthesis and metabolism have been cloned, allowing for the precise modulation of sphingolipid levels in this organism (30). In addition, *S. cerevisiae* synthesizes only three complex sphingolipids, inositolphosphorylceramide (IPC), mannose inositolphosphorylceramide (MIPC), and mannose diinositolphosphorylceramide (MIP₂C), making the interpretation of the physiological roles of sphingolipids much more straightforward (31).

Recently, we isolated the conditional *erg26-1* yeast mutant in a genetic screen designed to obtain sphingolipid metabolic mutants (32). Our biochemical studies demonstrated that *erg26-1* cells were defective in 4α-carboxysterol-C3 dehydrogenase activity, one of the three enzyme activities required for the conversion of 4,4-dimethylzymosterol to zymosterol. The defect in 4α-carboxysterol-C3 dehydrogenase activity in *erg26-1* cells caused the abnormal accumulation of specific zymosterol intermediates and had an effect on neutral and phospholipid biosynthesis and metabolism. We now
have gone back and examined in detail how the erg26-1 mutation affects sphingolipid biosynthesis and cell viability.
EXPERIMENTAL PROCEDURES

Strains, Media, and Miscellaneous Microbial Techniques - The yeast strains used in this study are derived from W303-1A (MATa leu2-3, 112 trp1-1 ura3-1 his3-11, 15 can1-100). Yeast strains were grown in either YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or in synthetic minimal media containing 0.67% Yeast Nitrogen Base (Difco) supplemented with the appropriate amino acids, and adenine. Yeast transformations were performed using the procedure described by Ito (33). For routine propagation of plasmids, E. coli XL1Blue cells were used and grown in LB medium supplemented with ampicillin (200 µg/ml).

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 pJR1133 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 genomic sequences subcloned into the various vectors were obtained by PCR amplification using the high fidelity pfu polymerase. All DNA sequences that were generated by PCR were sequenced and compared to the yeast genome database.

Metabolic Radiolabeling and Analysis of Lipids - Starting cultures for all lipid radiolabeling experiments were grown overnight at 27°C to exponential phase in synthetic complete media or
selective minimal media. OD$_{600}$ were then taken and cultures were diluted to 5x10$^6$ cells/ml and pre-incubated at 27 and 37˚C for 3 hours before antifungal treatment and/or radiolabeling. Radiolabeled lipids were resolved by TLC and visualized by autoradiography (Kodak XAR5). The percent 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, cultures were incubated with 5 µCi/ml [³H]myo-inositol for 30 min. For steady state labeling, cultures were incubated with 1µCi/ml [³H]myo-inositol for 5 hours. Radiolabeled sphingolipids were extracted with ethanol, water, diethyl ether, pyridine, 4.2 N ammonium hydroxide (15:15:5:1:0.018), treated with methylamine as described (38) and analyzed by one-dimensional TLC using chloroform, methanol, acetic acid, water (16:6:4:1.6). When necessary, wild type cultures were pre-incubated with specified antifungal compounds as described for the analysis of sterols (see below).

For pulse radiolabeling of ceramides, cultures were incubated with 5 µCi/ml [³H]dihydrosphingosine for 20 min. For steady state radiolabeling, 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 hours. 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 (15:15:5:1:0.018) and analyzed by one-dimensional TLC using chloroform, methanol, acetic acid, (95:4.5:0.5) (23).

For pulse labeling of sterols, antifungal-treated cultures were incubated at 30˚C with 1µCi/ml [¹⁴C]acetate for 30 min. For steady state labeling of sterols, cultures were incubated at 30˚C with 5µCi/ml [¹⁴C]acetate for 4 hours. Radiolabeled sterols were extracted using chloroform, methanol (2:1) and analyzed by one-dimensional TLC using petroleum ether, diethyl ether, acetic acid (70:30:1).

In all cases, cultures were pre-incubated for 1 hour in the absence and presence of a specified
concentration of antifungal compound (40 ng/ml fenpropimorph, 2 µg/ml fluconazole, 2 µg/ml terbinafine) prior to radiolabeling cells for sterol analysis.

For pulse labeling of phospholipids, cultures were incubated with 50 µCi/ml $[^{32}P]$ orthophosphate for 20 min. For steady state radiolabeling, cultures were incubated with 25 µCi/ml $[^{32}P]$ orthophosphate for several generations. Radiolabeled phospholipids were extracted using the spheroplast method of Atkinson et al., (39) and analyzed by one-dimensional TLC as described previously (38).

**GC/MS Analysis of Fatty Acids** - To analyze total fatty acids, cells were cultured at 27°C in synthetic media to a density of approximately 1 x 10^7 cells/ml. Cells were harvested by centrifugation and resuspended in an equivalent volume of fresh media prewarmed at 27°C (control cultures) or 37°C. Incubation of all cultures continued for three additional hours at the indicated temperatures. Cells were harvested again and washed with sterile water to remove residual media. Fatty acid methyl esters were prepared by HCl-methanolysis of whole cell lipids (40). Whole cells were collected by centrifugation in glass screw-capped test tubes. All residual water was removed by aspiration and the cell pellets were resuspended in 1 ml of 1 M methanolic HCl, 5% 2,2-dimethoxypropane (v/v). Test tubes were purged with nitrogen and incubated at 85°C for one hour. Fatty acid methyl esters were extracted from cooled samples by addition of 1 ml hexane, ethyl ether (1:1, v/v) and 1 ml 0.9% NaCl. Samples were vigorously mixed and then centrifuged to advance phase separation. The organic phase was collected and passed over a silica gel min-column preconditioned with hexane, ethyl ether (1:1, v/v) solvent and then washed with a minimal amount (1-2 ml) of solvent: The column flow-through was dried under nitrogen and dried fatty acid methyl esters were resuspended in 200 µl hexane. Samples of fatty acid methyl esters were analyzed by gas-liquid chromatography on a Hewlett-Packard model 6890 gas chromatograph using a 30 m HP-5 column. Instrument conditions were as follows: injection port temperature 220°C, detector temperature 300°C, initial oven temperature at 120°C. After a 1 min hold
at 120°C, the oven temperature was increased at 2°C/min up to a temperature of 150°C followed by a 4°C/min increase to a final temperature of 300°C and held for 5 min. Identification of cellular fatty acid species was by comparison of retention times to those of standard compounds and by GC-MS analysis using database files of fatty acid methyl ester spectra. GC-MS analysis was performed on a Hewlett-Packard model 6890 gas chromatograph and Hewlett-Packard model 5973 Mass Selective Detector. Run conditions were identical to that described above for gas-liquid chromatography.
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 Figure 2.

Using [H³]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 (23,41,42), 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 to wild type cells grown under the exact conditions (Fig. 2, ERG26 vs. erg26-1, pulse 27°C). IPC-B, MIPC, MIP₂C biosyntheses were all unaffected in erg26-1 cells grown at this temperature.

Strikingly, we found that IPC biosynthesis was almost completely shutdown in temperature-shifted erg26-1 cells. The biosynthetic levels of the three predominant IPC species detected in erg26-1 cells at the permissive temperature, IPC-B, IPC-C, and IPC-D, were all drastically reduced in temperature-shifted erg26-1 cells (Fig. 2, ERG26 vs. erg26-1, pulse 37°C). In contrast, we observed only a slight decrease in MIPC biosynthesis (2.3-fold) and a slight increase in the biosynthesis of MIP₂C in temperature-shifted erg26-1 cells (1.4-fold) (Fig. 2, ERG26 vs. erg26-1, pulse 37°C).

When we examined steady-state sphingolipid composition, we found that erg26-1 cells harbored defects in their ability to sustain normal levels of the major hydroxylated IPC species, IPC-B,
IPC-C, and IPC-D (Fig. 2, steady-state). A decrease in the levels of these three IPC species was detected at both temperatures in erg26-1 cells (Fig. 2, steady-state, 27°C & 37°C). In the case of IPC-B, we did not detect any accumulation of this complex sphingolipid in erg26-1 cells at either temperature. Whereas, the steady-state levels of IPC-C were decreased 15-fold and 7.3-fold, while IPC-D levels were decreased 6.2-fold and 4-fold, at 27°C and 37°C, respectively. On the other hand, steady-state MIPC levels were only slightly decreased (1.8-fold, 27°C; 2.0-fold, 37°C), whereas MIP2C levels were not affected in these cells at either temperature.

**erg26-1 Cells Contain Lower Levels of Phytosphingosine-derived Ceramides.** To ascertain what step(s) in the sphingolipid pathway is regulated by sterol levels in erg26-1 cells, we determined the rates of biosynthesis and metabolism of the hydroxylated ceramides using [H³]dihydrosphingosine radiolabeling and TLC analysis (23). Using this method, we were able to determine the biosynthetic and metabolic levels of four of the five hydroxylated ceramide species known to be synthesized in yeast cells (Fig. 1).

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 hydroxylase, Scs7p (Fig. 1) (23). The rate of biosynthesis and steady state levels of ceramide-B and -C were most severely reduced at the restrictive growth temperature (Fig. 3B & 3D). 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 dihydrosphingosine-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 can not say whether the biosynthetic and/or metabolic level of this ceramide species was affected by the erg26-1 mutation.
Cells Have Defects in Fatty Acid Biosynthesis- C\textsubscript{26} fatty acids are used exclusively to synthesize complex sphingolipids in yeast (43). We reasoned that the defects seen in complex sphingolipid metabolism in \textit{erg26-1} cells may cause the accumulation of C\textsubscript{26} 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 \textit{erg26-1} cells at the permissive and restrictive temperatures using GC/MS analysis.

We found that at either temperature, \textit{erg26-1} cells contained dramatically less C10:0 and C12:0 fatty acid species, while over accumulating C26:0 fatty acid (Fig. 4A). \textit{erg26-1} cells had 4- and 3-fold reductions in C10:0 and C12:0 fatty acids, while accumulating approximately 3-fold higher C26:0 fatty acid at both growth temperatures. In addition, we found that \textit{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 \textit{erg26-1} and wild type cells at both temperatures (Fig. 4B).

Altering Long Chain Base Levels in \textit{erg26-1} Cells Affects Cell Viability - The loss of proper ceramide-B levels in \textit{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 \textit{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 \textit{erg26-1} viability. In particular, we were interested in determining how the increased gene dosage of the \textit{SUR2} gene required for the biosynthesis of the LCB phytosphingosine affected \textit{erg26-1} viability (23,41).

Wild type and \textit{erg26-1} cells carrying individual high copy plasmids overexpressing \textit{SUR2}, \textit{SCS7}, or \textit{CCC2}, from the constitutive GPD promoter were examined for their ability to grow at the permissive and restrictive growth temperatures. \textit{SUR2} and \textit{SCS7} encode for sphingolipid hydroxylases (23,41), while \textit{CCC2} encodes for a copper transporter that is required for Cu\textsuperscript{2+} uptake (Fig. 1) (42).
Beeler et al., (48) have shown that the synthesis of fully hydroxylated IPC-D is dependent on $\text{CCC}2$ and the concentration of $\text{Cu}^{2+}$ in the media.

As shown in Figure 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, panels A & C). In contrast, the constitutive overexpression of either $\text{SUR}2$ or $\text{CCC}2$ alone in $\text{erg}26-1$ cells was highly toxic to cell growth at the permissive temperature of 27°C (Fig. 5, $\text{erg}26-1$, panel B). Whereas the overexpression of $\text{SCS}7$ had no effect on cell growth at the permissive temperature, and actually suppressed the $ts$ defect of $\text{erg}26-1$ cells (Fig. 5, $\text{erg}26-1$, panels B & C).

Based on these results, we predicted that the constitutive overexpression of $\text{SUR}2$ increased LCB and/or LCBP levels in $\text{erg}26-1$ cells, since these cells are defective in ceramide-B metabolism. This suggests that accumulation of one or both of these LCBs may be responsible for the $\text{SUR}2$-dependent cell toxicity observed at low temperatures. To determine whether the increased accumulation of LCB or LCBP contributes to the observed $\text{SUR}2$-dependent growth defect of $\text{erg}26-1$ cells, we constructed $\text{erg}26-1$ strains lacking either the $\text{LBP}1/\text{YSR}2/\text{LCB}3$ or $\text{LBP}2/\text{YSR}3$ phosphatase genes (46,49,50) and determined their growth viability when $\text{SUR}2$ was overexpressed.

Using these strains, we found that the loss of $\text{LBP}1$, but not $\text{LBP}2$, remediated the $\text{SUR}2$-dependent cell toxicity of $\text{erg}26-1$ cells at low temperatures (Fig. 6, panels A, B, & C). In fact, the loss of $\text{LBP}1$ suppressed the $ts$ phenotype of $\text{erg}26-1$ cells (Fig. 6, panel D). Interestingly, the loss of $\text{LBP}2$ in $\text{erg}26-1$ cells resulted in drastically less growth at low temperatures, similar to that seen in $\text{erg}26-1$ cells overexpressing $\text{SUR}2$ (Fig. 6, panels A vs. C; $\text{erg}26-1$ 2µ vs. $\text{erg}26-1$ lbp2 2µ). $\text{lbp}1$-dependent suppression of $\text{SUR}2$-dependent toxicity was not acting through decreasing the accumulation of toxic zymosterol intermediates, as these lipids accumulated to similar levels in all strains (Fig. 6, panel E).

Together our genetic results point to the accumulation of LCB contributing to $\text{SUR}2$-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 define (i) 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, as *erg26-1 sur2* cells overexpressing *SCS7* remained viable at high temperature (Fig. 7A vs. 7B). 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* vs. *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 Cu$^{2+}$, Mn$^{2+}$, Zn$^{2+}$,
and Co\(^{2+}\). \textit{erg26-1} cells were sensitive only to the addition of Cu\(^{2+}\) in the media, while demonstrating wild-type like sensitivity to Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\) supplementation (Fig. 8B vs. 8C-E). The increased Cu\(^{2+}\) sensitivity could be remediated by the addition of the copper chelating agent bathocuproine (not shown).

\textit{Cells Harboring Specific Blocks in Sterol Biosynthesis Harbor Sphingolipid Defects} – If yeast do possess mechanisms which 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 \([^{3}H]\)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, \textit{pulse}), which led to the sustained increased accumulation of steady-state levels of IPC-B in drug-treated cells (Fig. 9, \textit{steady-state}). In addition, we found that there was a reduction in the biosynthesis of IPC-C and MIPC (Fig. 9, \textit{pulse}). The biosynthetic rate of production of these sphingolipids was reduced approximately 3.5-fold. This was not due to a decrease in the levels of the IPC precursor PI, as drug-treated cells had PI levels that were comparable to 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 to wild type cells (Fig. 9, \textit{steady-state}). And these cells had lower sustained levels of IPC-D (1.7-fold lower than wild type) (Fig. 9, \textit{steady-state}).

Later blocks in sterol biosynthesis also perturbed sphingolipid metabolism, as evidenced by the sphingolipid defects detected in \textit{erg2} and \textit{erg6} cells lacking sterol C-8 isomerase or sterol C-24 methyltransferase activities, respectively (52). \textit{erg2} cells synthesized little IPC-C and had lower biosynthetic levels of IPC-D within the timeframe of our pulse assay (Fig. 9, \textit{pulse}). In addition, the
sustained levels of IPC-C remained lower in steady-state labeled erg2 cells (Fig. 9, steady-state). Moreover, erg6 cells synthesized lower levels of MIPC (Fig. 9, pulse). However, the metabolic level of MIPC in these cells was comparable to wild type cells (Fig. 9, steady-state).
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 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, 4α-carboxysterol-C3 dehydrogenase, is one of three enzyme activities required for proper zymosterol 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, as 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 synthases (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, dihydrosphingosine-derived ceramide-A metabolism was far less affected (Fig. 3). This brings up the question of how might sterol levels preferentially effect 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 at 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. Regulation of ceramide biosynthesis may also be at the level of transcription

\[ csg2 \] mutants which are defective in MIPC biosynthesis are sensitive to calcium (63). Dunn \textit{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 antifungals (41). How altering the hydroxylation-state of sphingolipids causes, or is responsible for, the regulation of calcium homeostasis or antifungal 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 ORFs \( YDL222 \) and \( YNE194 \) (65). Sur7p, Ydl222p, 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 can not 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 temperature and are totally lacking these patches at high temperature\(^1\). 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 Scs7p in \textit{erg26-1} cells.

LCB and LCBP affect processes in yeast including calcium signaling (66), endocytosis (67), the 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 \textit{erg26-1} cell viability. Our genetic studies suggested that lowering the level of LCB or increasing the level of LCBP increased \textit{erg26-1} cell growth under certain conditions. How might an increase in LCBP levels help \textit{erg26-1} cells to grow under stress conditions? One possibility is that accumulation of LCBP in \textit{erg26-1} cells activates a compensatory Ca\textsuperscript{2+}-dependent pathway. Birchwood \textit{et al.}, (66) recently showed that yeast mutants lacking \textit{LBP1/YSR2/LCB3} and the LCBP lyase gene \textit{DPL1} exhibited constitutively high Ca\textsuperscript{2+} accumulation and signaling. In the absence of \textit{LBP1}, \textit{erg26-1 lbp1} cells, because \textit{erg26-1} cells possibly accumulate LCB, may accumulate a level of LCBP that is similar to that seen in \textit{lbp1 dpl1} cells. Ca\textsuperscript{2+} supplementation has been shown to suppress the essential requirement for \textit{ERG24} C-14 sterol reductase function (69). Moreover, studies have shown that recessive mutations that suppress the essential requirement for \textit{ERG24} also suppress the calcium sensitivity of \textit{csg2} mutants (28,32). These results in combination with our findings would suggest that sterols directly or indirectly regulate calcium homeostasis in some way.
REFERENCES


Footnotes:


2 Donohue, T., Vermitsky, J.P., and Nickels, J.T., manuscript in preparation.
**Abbreviations:** SRE, sterol response element; SREBP, sterol response element binding protein; IPC, inositolphosphorylceramide; MIPC, mannose inositolphosphorylceramide; MIP$_2$C, mannose diinositolphosphorylceramide; LCB, long chain sphingoid base; LCBP, long chain sphingoid base phosphate, TLC, thin layer chromatography; VLCFA, very long chain fatty acid.
FIGURE LEGENDS

Fig. 1. **Biosynthetic pathway for the production of hydroxylated ceramides in yeast.** * denotes the hydroxyl group added during the biosynthetic reaction. Adapted from (23).

Fig. 2. **erg26-1 cells harbor defects in sphingolipid biosynthesis and metabolism.** For pulse and steady-state labeling of sphingolipids, wild type (ERG26) and erg26-1 cells grown in synthetic media were shifted to 27° C or 37° C and incubated with [3H]inositol as described in “Experimental Procedures”. Radiolabeled sphingolipids then were extracted and deacylated. The deacylated sphingolipid fraction was resolved by one-dimensional TLC using chloroform, methanol, acetic acid, water (16:6:4:1.6). Figure 2 represents the typical results obtained from 5 independent experiments. Abbreviations used are: IPC-B, inositolphosphorylceramide-B species; IPC-C, inositolphosphorylceramide-C species; IPC-D, inositolphosphorylceramide-D species; MIPC, mannose inositolphosphorylceramide; MIP$_2$C, mannose diinositolphosphorylceramide.

Fig. 3. **erg26-1 cells have lower levels of phytosphingosine-derived ceramides.** The rates of biosynthesis (A & B) and steady-state levels (C & 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 in “Experimental Procedures”. Experiments were performed at the permissive (A & C) and restrictive (B & D) growth temperatures. Abbreviations used are: cer-A, ceramide-A; cer-B’, ceramide-B’; cer-B, ceramide-B; cer-, ceramide-C. The LCB backbone of ceramide-A is dihydrosphingosine. Ceramide B’ is derived from cer-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 cer-B and contains an additional hydroxyl group attached to the fatty acid moiety.

**Fig. 4.** *erg26-1* cells harbor defects in fatty acid metabolism. Steady-state levels of various fatty acid species were determined for wild type and *erg26-1* cells using GC/MS. Wild type cells, 27°C, *black bars*; *erg26-1* cells, 27°C, *white bars*; Wild type cells, 37°C, *dark gray bars*; *erg26-1* cells, 37°C, *light gray bars*.

**Fig. 5.** Altering individual sphingolipid hydroxylase activities affects *erg26-1* cell growth and viability. Wild type (*W303-1A*) and *erg26-1* cells harboring 2 µ plasmids overexpressing *CCC2*, *SCS7*, or *SUR2* from the constitutive GPD promoter were streaked onto selective synthetic plates and grown at 27°C and 37°C for 72 and 48 hr, respectively.

**Fig. 6.** The loss of the LCB phosphatase gene, *LBP1*, remediates the cell toxicity associated with overexpression of the LCB hydroxylase gene, *SUR2*. *Panels A, B, and C*, Various strains were streaked onto selective synthetic plates and grown at 27°C for 72 hr. *Panel D*, Various strains were streaked onto selective synthetic plates and grown at 27°C and 37°C for 72 and 48 hr, respectively. *Panel E*, *erg26-1*, *erg26-1 lbp1*, and *erg26-1 lbp2* cells were steady-state radiolabeled with [14C]acetate at the indicated temperatures and sterols were extracted and resolved by TLC.

**Fig. 7.** Sur2p-dependent LCB hydroxylation is not required for *SCS7*-dependent *ts* suppression of *erg26-1* cells. Strains harboring 2µ or 2µ-*SCS7* plasmids were streaked onto selective plates and grown at 27°C (*panel A*) and 37°C (*panel B*) for 72 and 48 hr, respectively.
Fig. 8. The accumulation of IPC-D most likely plays a role in CCC2-dependent cell toxicity of erg26-1 cells. Panel A, Strains harboring 2μ or 2μ-CCC2 plasmids were streaked onto selective plates and grown at 27° C for 72 hr. Panels A, B, and C, erg26-1 cells were grown at 27° C for 60 hr in liquid media supplemented with the indicated concentrations of divalent cations. Plate viability assays were performed to determine the number of viable cells.

Fig. 9. Additional blocks in sterol biosynthesis result in sphingolipid metabolic regulation. Wild type, erg2, and erg6 cells were pulse or steady-state radiolabeled with [3H]inositol and sphingolipids were extracted and resolved as described in Figure 2. For the antifungal studies, wild type cells were pretreated with drug for 1 hr prior to radiolabeling.
Acknowledgements

This work was supported by a National Institutes of Health Grant HL67401-01A1 (JN), a Basil O’Connor Starter Scholarship Award (J.N) and an Atorvastatin Research Award (VMM) sponsored by Pfizer Inc. We thank the Hope College Department of Chemistry for the use of the gas chromatograph and gas chromatograph-mass spectrograph, and Drs. E. Sanford and W. Mungall for their technical assistance in their operation. We are grateful to Dr. Tom Edlind and members of his laboratory for many helpful discussions. We thank Drs. Valeria Culotta, Chris Beh, and Jasper Rine for plasmids.
Figure 2

Pulse

27°

ERG26  erg26-1  ERG26  erg26-1

37°

Steady-State

27°

ERG26  erg26-1  ERG26  erg26-1

37°

IPC-B
IPC-C
IPC-D
MIPC

MIP2C
ORIGIN
Figure 3
Figure 4

A

% Total Fatty Acid

Temp. FA sp. 27° 37° 27° 37° 27° 37° 27° 37° 27° 37°

10:0 12:0 14:0 16:0 18:0

B

% Total Fatty Acid

Temp. FA sp. 27° 37° 27° 37° 27° 37° 27° 37°

16:1 18:1
Figure 5

```
W303-1A                  erg26-1

27°  A                  27°  B

C                  D

37°

2μ        CCC2

SUR2        SCS7
```
Figure 8

(B) [CuCl$_2$] mM vs. OD$_{600}$

(C) [ZnCl$_2$] mM vs. OD$_{600}$

(D) [CoCl$_2$] mM vs. OD$_{600}$

(E) [MnCl$_2$] mM vs. OD$_{600}$
Figure 9

Pulse

WT  Terbinafine  Fluconazole  erg2  erg6
PI  IPC-B  IPC-C  IPC-D  MIPC

Steady-State

WT  Terbinafine  Fluconazole  erg2  erg6
PI  IPC-B  IPC-C  IPC-D  MIPC  Lyso-PI

MIP2C  ORIGIN
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J. Biol. Chem. published online May 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204115200

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