The Effect of the erg26-1 Mutation on the Regulation of Lipid Metabolism in Saccharomyces cerevisiae*

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A temperature-sensitive Saccharomyces cerevisiae mutant harboring a lesion in the ERG26 gene has been isolated. ERG26 encodes 4α-carboxysterol-C3 dehydrogenase, one of three enzymatic activities required for the conversion of 4,4-dimethylzymosterol to zymosterol. Gas chromatography/mass spectrometry analyses of sterols in this strain, designated erg26-1, revealed the aberrant accumulation of a 4-methyl-4-carboxy zymosterol intermediate, as well as a novel 4-carboxy-sterol. Neutral lipid radiolabeling studies showed that erg26-1 cells also harbored defects in the rate of biosynthesis and steady-state levels of mono-, di-, and triglycerides. Phospholipid radiolabeling studies showed defects in the rate of biosynthesis of both phosphatidic acid and phosphatidylinositol. Biochemical studies revealed that microsomes isolated from erg26-1 cells contained greatly reduced 4α-carboxysterol-C3 dehydrogenase activity when compared with microsomes from wild type cells. Previous studies have shown that loss of function mutations in either of the fatty acid elongase genes SUR4/ELO3 or FEN1/GNS1/ELO2 can “bypass” the essentiality of certain ERG genes (Ladeveze, V., Marcireau, C., Delourme, D., and Karst, F. (1993) Lipids 28, 907–912; Silve, S., Leplatois, P., Josse, A., Dupuy, P. H., Lanau, C., Kaghad, M., Dhers, C., Picard, C., Rahier, A., Tatón, M., Le Fur, G., Caput, D., Ferrara, P., and Loison, G. (1996) Mol. Cell. Biol. 16, 2719–2727). Studies presented here have shown that this sphingolipid-dependent “bypass” mechanism did not suppress the essential requirement for zymosterol biosynthesis. However, studies aimed at understanding the underlying physiology behind the temperature-sensitive growth defect of erg26-1 cells showed that the addition of several antifungal compounds to the growth media of erg26-1 cells could suppress the temperature-sensitive growth defect. Fluorescence microscopic analysis showed that GFP-Erg26p and GFP-Erg27p fusion proteins were localized to the endoplasmic reticulum. Two-hybrid analysis indicated that Erg25p, Erg26p, and Erg27p, which are required for the biosynthesis of zymosterol, form a complex within the cell.

The budding yeast Saccharomyces cerevisiae is an excellent model system for studying sterol biosynthesis and regulation (1, 2). The sterol biosynthetic pathway in this yeast is highly conserved with its mammalian counterpart. The difference being that ergosterol is synthesized as the end product sterol rather than cholesterol. All of the structural genes have been cloned that are required for the biosynthetic steps necessary to synthesize sterols in S. cerevisiae (1, 2). In addition, many of the enzyme activities involved in yeast sterol synthesis have been characterized with respect to their enzymological parameters (3–7). Moreover, some physiological functions for yeast sterols have been assigned using constitutive sterol mutants (2, 8, 9).

One of the later steps in the sterol pathway is the conversion of 4,4-dimethylzymosterol to zymosterol (1). The reaction is carried out in two sequential demethylation reactions by three separate enzyme activities. In mammalian and plant cells, the activities required, a C-4 methyl oxidase, a 4α-carboxysterol-C3 dehydrogenase (C-4 decarboxylase) (4α-CD), and a C-3 ketoreductase, have been characterized from partially purified preparations (10–15). These analyses have given an excellent insight into the steps involved in C-4 demethylation. The first step is initiated by the C-4 methyl oxidase, whereby this enzyme converts the C-4α methyl group of 4,4-dimethylzymosterol to alcohol and aldehyde intermediates and then to a carboxylic acid. Next, the carboxyl group is removed by the 4α-CD, with epimerization of the 4β-methyl group to a 4α stereochrome and a 3-keto group being formed during this step. Finally, the 3-keto group is reduced to a β-hydroxy sterol by the 3-ketoreductase. The next round of demethylation is initiated once the second methyl group translocates to the C-4α position.

In S. cerevisiae, the C-4 methyl oxidase, 4α-CD, and C-3 ketoreductase required for the demethylation of 4,4-dimethylzymosterol are encoded for by the ERG25 (16), ERG26 (17), and ERG27 (18) genes, respectively. All three genes are essential under aerobic growth conditions. However, viable single null mutants have been constructed by genetic means. These strains have been very useful in determining the changes in sterol composition due to the individual loss of function of these genes. Yeast strains lacking the ERG25 gene accumulate the

* This work was supported by Mid-Atlantic American Heart Association Grants 00511027 and 9805529U (to J. N.) and by the March of Dimes Foundation Basil O'Connor Starter Scholarship Grant FY99–277 (to J. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: 4α-CD, 4α-carboxysterol-C3 dehydrogenase; 5-FOA, 5-fluoroorotic acid; PCR, polymerase chain reaction; Yip; Yeast Integrating plasmid; GC/MS, gas chromatography/mass spectrometry; CDP-DG, CDP-diacylglycerol; t.s., temperature-sensitive; ER, endoplasmic reticulum; GFP, green fluorescence protein; DG, diacylglycerides; MG, monoglycerides; TG, triglycerides; PI, phosphatidylinositol; PA, phosphatidic acid.
substrate of the Erg25p, 4,4-dimethylzymosterol (16), whereas strains lacking ERG26 accumulate the expected 4-carboxy-bromo-5-dehydro-3-keto sterols if they are fed lanosterol, a sterol intermediate that is an upstream precursor of the substrate for the Erg27p-directed 3-ketoreductase reaction.

A yeast erg25 mutant has been isolated through a screen selecting for mutants unable to grow on low iron media (19). Interestingly, exogenous iron transport activity in this erg25 mutant is normal, suggesting that the changes in zymosterol intermediate levels seen in these cells are not affecting iron uptake but another intracellular process. In mammalian cells, a cholesterol auxotrophic Chinese hamster ovary cell line has been isolated that accumulates carboxysterols (20, 21). The lesion responsible for the sterol metabolic defect is thought to reside within the gene encoding for 4α-CD activity, because this activity is almost completely lacking in these cells.

In this report, we describe the isolation and initial characterization of the erg26-1 conditional growth mutant. By using various analytical and biochemical methods, we demonstrate that erg26-1 cells are defective in 4α-CD activity and in zymosterol biosynthesis, whereby they accumulate aberrant zymosterol intermediates. Additional lipid metabolic labeling studies show that erg26-1 cells also harbor defects in neutral lipid and phospholipid biosynthesis and metabolism. Furthermore, by using specific pharmacological agents, we demonstrate that the conditional growth phenotype of erg26-1 cells is due to the toxic accumulation of zymosterol intermediates at high temperatures rather than the loss of ergosterol biosynthesis. Further studies show that this toxic phenotype cannot be suppressed by mutations in sphingolipid biosynthesis that previously have been shown to suppress ERG gene essentiality (22, 23). Finally, by using fluorescence microscopy and two-hybrid analysis, we demonstrate that Erg26p and Erg27p both localize to the endoplasmic reticulum and that Erg25p physically associates with Erg26p and Erg27p within the cell.

EXPERIMENTAL PROCEDURES

Isolation of Sphingolipid Metabolic Mutants—W303-1A was mutagenized with ethylmethane sulfonate by standard procedures (30) and grown on inositol-free synthetic media for 3 days at 25 °C. Colonies were transferred and grown on filter paper replicas and labeled with 100 μCi of [3H]inositol, essentially as described previously (31). After labeling for 40 min at 30 °C, the cells were washed three times with 5% trichloroacetic acid, air-dried, and exposed to Kodak BioMax MR film for 6 days. Following exposure, the filters were treated sequentially with the following solvents to chemically deacetylate and remove the [3H]inositol incorporated into phosphatidylinositol.0.2 n KOH in methanol/toluene (1:1, v/v) at room temperature for 60 min, methanol (10 min), acetic acid (10 min), and twice more in methanol for 10 min each. Filters were dried, sprayed with ENHANCE (PerkinElmer Life Sciences), and exposed to Kodak X-Omat film for 18 h. The signal intensity for each colony before and after deacetylation was compared, and isolates that had reduced signal after deacetylation relative to their initial signal were selected and grown. The entire labeling procedure was repeated on the selected colonies as described, except radioactivity was quantified on a Molecular Dynamics PhosphorImager using a tritium-sensitive screen.

Cloning the ERG26 Gene—Sphingolipid metabolic mutant H21 was transformed by a YCP50-based yeast genomic low copy library (32). Transformed H21 cells were plated onto synthetic plates lacking uracil, and plasmids harboring yeast sequences conferring temperature resistance were selected. Plasmid YCp50-16-1 was used as a representative suppressor plasmid (Fig. 1A) based on restriction analyses of 12 independent suppressor plasmids. DNA sequencing analysis and subcloning experiments revealed that the ERG26 gene was responsible for conferring temperature resistance to H21. Linkage analysis was used to confirm that the ERG26 gene was mutated in the H21 mutant strain and that the mutation was recessive. Several backcrosses were performed on the H21 strain to obtain the haploid segregant designated erg26-1. The ts and lipid metabolic phenotypes of erg26-1 are identical to that of H21.

Labeling and Analysis of Phospholipids, Neutral Lipids, and Sterols—Starting cultures for all labeling experiments were derived from exponential cells grown at 23 °C in the described media. For pulse labeling of phospholipids, cells grown in synthetic media were shifted to 37 °C and incubated with 50 μCi/ml [3H]thiocoliphosphate for 20 min. For steady-state labeling of phospholipids, 0.5A590/ml cells grown in synthetic media were shifted to 37 °C and incubated with 10 μCi/ml [3H]thiocoliphosphate for at least six generations. Phospholipids were extracted using the spheroplast method of Atkinson and Henry (33) and analyzed by one-dimensional TLC as described previously (34). For pulse and steady-state labeling of neutral lipids using ts cells grown in synthetic media were shifted to 37 °C and incubated with 1 μCi/ml [14C]acetate for 30 min or 4 h, respectively. Radiolabeled neutral and sterol lipids were extracted using chloroform, methanol (2:1) and analyzed by one-dimensional TLC using hexane, ethyl ether, acetic acid (80:20:2) and petroleum ether, diethyl ether, acetic acid (70:30:1), respectively (35). In all cases, radiolabeled lipids were visualized by autoradiography using an x-ray film (Kodak XAR 5). The percent incorporation of each lipid species was determined by densitometry using a Bio-Rad Model GS-670 Imaging Densitometer and Molecular Analyst Software Version 1.4.1.

Preparation of Microsomes for the 4α-Carboxylated-C3 Dehydrogenase Assay—Yeast cells were disrupted by glass bead homogenization (0.45-mm diameter) in 100 mM phosphate buffer (pH 7.5) containing 15 mM reduced glutathione and 30 mM nicotinamide for 10 min at 2 °C.

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4α-Carboxylated-C3 Dehydrogenase Assay—Microsomes (0.4 ml = 1 mg of protein) were incubated in the presence of 100–200 μM exogenous synthetic 4α-carboxy-5a-cholest-7-en-3β-ol that had been emulsified in 1 g/liter Tween 80, and 200 μM NAD+−. Incubations were continued aerobically at 30 °C with gentle stirring for 50 min. The reaction was stopped by adding 0.5 ml of 6% KOH/EtOH. After addition of a known amount of coprostanone (2–10 μg) as an internal standard, the sample was reduced twice with 10% Na2SO3, the extract was extracted three times with a total volume of 15 ml of n-hexane. After drying with Na2SO4, the extract was concentrated to dryness and analyzed by TLC on silica gel eluted with CHCl3/CH3OH (2:1) (two developments). The fraction migrating as authentic standards of coprostanone and 3α,5β-cholenoate and containing the enzymatically produced 3α-cholenoate (RF = 0.50–0.70) was eluted and analyzed by GLC using a fused-silica capillary column DB1 (240–280 °C, 2 °C/min).
and hydrogen as the carrier gas. The amount of Δ'-cholestenone produced (tR = 1.11) was calculated from the amount of coprostane (tR = 1.0) and the area of their respective peaks, allowing the rate of the reaction to be determined. The ketone metabolite and other endogenous sterones (if present) were identified by capillary GLC and coupled GLC-MS analysis. The ketone metabolite produced by the reaction was thus unequivocally identified as cholest-7-en-3-one by coincidental retention time in GLC and by an electron impact spectrum identical to that of an authentic synthetic standard. The 4α-carboxy-7-en-3β-ol substrate was synthesized as described previously (15).

**GC/MS Analysis of Sterols—** Yeast cells were hydrolyzed in 6 N ethanolic NaOH and extracted exhaustively with ethyl acetate. The extract was treated with diazomethane to form the methyl esters of any free carboxyl groups and then with Sil-Prep (Supelco) to form the trimethylsilyl ethers of free hydroxyl substituents. The sample was then dissolved in hexane and injected onto a 30-m HP-5MS capillary column (Agilent Technologies) installed in an HP 6890 gas-chromatograph interfaced with an HP 5972A mass selective detector. After the injection, the column was kept at 100 °C for 2 min then raised to 285 °C at a rate of 35 °C/min. The carried gas was helium at a flow rate of ~1 ml/min. For the analysis of the reaction products formed in the 4α-carboxysterol-C3 dehydrogenase assay, MS and GLC/MS were determined at 70 eV with a Fisons MD800 spectrometer. The GLC separation was carried out with a Varian 3400 CX GLC instrument equipped with a flame ionization detector and a fused-silica capillary column (30-m length; 0.32-mm inner diameter) × 0.25-μm film coated with DB5 (H2, flow 2 ml/min). The temperature program used included a 30 °C/min rise from 60 to 240 °C followed by a 2 °C/min rise from 240 to 280 °C. Relative retention times (tR) are given with respect to cholesteryl (tR = 1).

**Antifungal Supplementation Assays—** 1 × 107 cells were spotted onto YEFP plates lacking or containing the indicated antifungal compounds. The plates were then incubated at either 23 or 37 °C for 4 days. Alternatively, 2-ml cultures of various yeast strains were grown to 0.5–1.0 A600 at 23 °C and subsequently inoculated at 1.0 × 107 cells/ml into 96-well microtiter dishes containing YEFP and various concentrations of antifungal compounds. Cells were then allowed to grow at either 23 or 37 °C for several days without shaking, and the cell number was determined by plate viability assays. The degree of reduction in the accumulation of sterol intermediates due to antifungal supplementation was determined by [14C]acetate and TLC analyses.

**RESULTS**

**Isolation and Characterization of a Yeast Sterol Biosynthesis Mutant Having a Defect in 4α-Carboxysterol-C3 Dehydrogenase Activity—** A screen was initiated to isolate yeast mutants having defects in sphingolipid metabolism. Because *S. cerevisiae* cells incorporate inositol into complex sphingolipids, the strategy was to look for temperature-sensitive (ts) loss of function mutations that reduced or eliminated the incorporation of [3H]inositol into the yeast cell membrane. Among the ts mutants obtained, a sphingolipid biosynthetic mutant was isolated whose ts phenotype was suppressed in low copy by the ERG26 gene (Fig. 1A). ERG26 is believed to encode for the enzyme 4α-carboxysterol dehydrogenase. ERG26 mutant strains were constructed by transforming the ERG26 gene on a low copy plasmid (Fig. 1B). Linkage analysis was performed in this mutant, designated H21, and showed that the mutation responsible for the ts defect resided within the ERG26 gene (Fig. 2A). H21 was backcrossed several times to the parental W303-1A wild-type strain and an erg26-1 haploid segregant was generated. We used this erg26-1 strain to examine in greater detail the defects in sterol lipid biosynthesis brought about by mutating ERG26 and to determine whether neutral lipid and phospholipid biosynthesis were also perturbed.

We first analyzed the sterol lipid composition of wild type and erg26-1 mutant cells grown at the nonpermissive temperature. GLC analyses of these extracts revealed the presence of several lipid peaks, with peaks 1 through 5 being detected in both wild type and erg26-1 cells (Fig. 2, A and B). However, there were three additional peaks detected in erg26-1 cells that were not seen in wild type cells (Fig. 2, A versus B). The three peaks, designated 6, 7, and 8, had retention times of 35.09, 38.73, and 39.32 min, respectively (Table I). The molecular structures of all of the peaks detected by GC are outlined in Table I and are based on our MS analyses. Using the MS data obtained, we identified peaks 1, 2, 4, and 5 as ergosterol, fecosterol, lanosterol, and 14-methylfe-costerol, respectively. We were unable to predict the molecular structure of peak 3.

The MS spectra of peaks 6 and 7 are shown in Fig. 3. They are consistent with these sterol intermediates being the methyl esters of a 4-methyl-4-carboxy-diunsaturated sterol (Fig. 3A, peak 7) and a 4-carboxy-diunsaturated sterol (Fig. 3B, peak 6). Based on the reaction mechanism involved in the demethylation of 4,4-dimethylzymosterol, we believe that these interme-

**Fig. 1. Low copy ERG26 suppresses the ts growth and sterol biosynthetic defects of erg26-1 cells.** A, various ERG26 strains were streaked onto Ura−plates and grown for 48 h at the indicated temperatures. B, various ERG26 strains were labeled with [14C]acetate for several generations. The total lipid fraction was isolated by chloroform, methanol extraction (2:1). Sterols were resolved by TLC using the solvent system petroleum ether, diethyl ether, and acetic acid (70:30:2). Radio-labeled sterols were detected by autoradiography. In the case of the ERG26 wild-type strain and the erg26-1 strain carrying the plasmid pRS-ERG26, the levels of sterol intermediates accumulating at 37 °C are shown.
Wild type (\textit{A}) and \textit{erg26-1} (\textit{B}) cells were grown to exponential phase at 23°C. Cells were then shifted to 37°C for several generations. Sterol extracts were obtained and the methyl esters of all sterol intermediates accumulating in cells were synthesized using diazomethane. GC analysis of diazomethane-treated sterol extracts reveals the presence of aberrant sterol peaks. Peaks no. Retention time (min) and Known sterol or proposed sterol structure

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Retention time (min)</th>
<th>Known sterol or proposed sterol structure</th>
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<tbody>
<tr>
<td>1</td>
<td>21.37</td>
<td>Ergosterol</td>
</tr>
<tr>
<td>2</td>
<td>23.52</td>
<td>Fecosterol</td>
</tr>
<tr>
<td>3</td>
<td>23.85</td>
<td>Unknown</td>
</tr>
<tr>
<td>4</td>
<td>24.95</td>
<td>Lanosterol</td>
</tr>
<tr>
<td>5</td>
<td>25.95</td>
<td>14-Methyl-fecosterol</td>
</tr>
<tr>
<td>6</td>
<td>35.09</td>
<td>4-Carboxy-cholesta-8,24-dien-3-ol</td>
</tr>
<tr>
<td>7</td>
<td>38.73</td>
<td>4-Methyl-4-carboxy-cholesta-8,24-dien-3-ol</td>
</tr>
<tr>
<td>8</td>
<td>39.32</td>
<td>Epimer of peak 7</td>
</tr>
</tbody>
</table>

**Fig. 2.** GC analysis of diazomethane-treated sterol extracts from \textit{erg26-1} cells reveals the presence of aberrant sterol peaks. Wild type (\textit{A}) and \textit{erg26-1} (\textit{B}) cells were grown to exponential phase at 23°C. Cells were then shifted to 37°C for several generations. Sterol extracts were obtained and the methyl esters of all sterol intermediates accumulating in cells were synthesized using diazomethane. GC analysis of diazomethane-treated sterol extracts reveals the presence of aberrant sterol peaks.

**To begin to ascertain the biochemical basis for the accumulation of these sterol intermediates in \textit{erg26-1} cells, 4α-CD activity was assayed by introducing the wild type \textit{ERG26} gene on a low copy plasmid (Table I, \textit{erg26-1} pRS-\textit{ERG26}). Thus, \textit{erg26-1} cells do contain weakened 4α-CD activity.**

Using GC-MS analysis, we determined the molecular structures of the ketone products formed by the wild type and mutant Erg26p. Wild type Erg26p catalyzed the formation of the expected single product, cholest-7-en-3-one (15). On the other hand, we observed that the mutant \textit{erg26-1p} microsomal assay preparation contained additional compounds in the steron fraction from the TLC purification. MS spectra analysis of the sterones in this fraction was consistent with these sterones being the expected product, cholest-7-en-3-one, as well as other endogenous sterones, including 4α-methyl-cholest-8,24-dien-3-one and cholest-8,24-dien-3-one. These aberrant sterones were not seen in any significant amounts in the assays performed with wild type Erg26p. Based on these results, we conclude that the accumulation in \textit{erg26-1} cells of the zymosterol intermediates 4β-methyl-4α-carboxy-cholesta-8,24-dien-3β-ol and 4α-carboxy-cholesta-8,24-3β-ol is a direct result of a defect in 4α-CD activity encoded for by the \textit{ERG26} gene.

**erg26-1 Cells Have Defects in Neutral Lipid Metabolism**—To examine in greater detail whether the \textit{erg26-1} mutation caused additional perturbations in lipid metabolism, we determined the rates of biosynthesis and steady-state levels of neutral lipids and phospholipids in both wild type and \textit{erg26-1} cells. Experiments were carried out at both the permissive and non-permissive temperatures for \textit{erg26-1} cell growth. Under these conditions, \textit{erg26-1} cells were found to harbor several defects in the rate of biosynthesis and steady-state levels of neutral lipids. First, we found that the rates of biosynthesis (Fig. 4, \textit{A} and \textit{B}) and steady-state levels (Fig. 4, \textit{C} and \textit{D}) of ergosterol and sterol esters were reduced in \textit{erg26-1} cells when compared with wild type cells. The reduction in the levels of these sterols was seen in both the permissive (Fig. 4, \textit{A} and \textit{C}) and non-permissive (Fig. 4, \textit{B} and \textit{D}) temperatures for growth. However, the greatest decrease in the rate of biosynthesis of ergosterol (5-fold) and sterol esters (2.5-fold), and in the steady-state levels of ergosterol (1.8-fold) and sterol esters (3.0-fold), was seen in temperature-shifted \textit{erg26-1} cells (Fig. 4, \textit{B} and \textit{D}). In agreement with these data, we found a 4-fold decrease in ergosterol content in the microsomes of \textit{erg26-1} cells compared with the wild type microsomes.

Second, we found that \textit{erg26-1} cells grown at the non-permissive temperature had increased rates of biosynthesis of mono-(4.0-fold) and diglycerides (3.5-fold), whereas a decreased rate of biosynthesis of triglycerides (1.7-fold) (Fig. 4, \textit{A versus B}). The steady-state levels of these lipids in \textit{erg26-1} cells, however, were found to be comparable to those of wild type cells (Fig. 4, \textit{C versus D}). Finally, two \textit{[14C]}acetate-labeled lipids (Fig. 4, double asterisks) were found to accumulate in \textit{erg26-1} cells.
under all conditions examined. Their levels were found to drastically increase in temperature-shifted erg26-1 cells. They were extracted from the neutral lipid TLC plate and chromatographed under the conditions used for TLC analysis of sterols. They were found to comigrate with the 4β-methyl-4α-carboxy-cholesta-8,24-dien-3β-ol and 4α-carboxy-cholesta-8,24–3β-ol sterols seen in erg26-1 cells.

We also examined the rates of biosynthesis and steady-state levels of individual phospholipids under the same conditions as those described above. The only significant defect in phospholipid metabolism was found in temperature-shifted erg26-1 cells, where there was an increase in the rate of biosynthesis of PA (1.9-fold) and a decrease in the rate of biosynthesis of PI (1.7-fold) (Fig. 5, A versus B). However, the steady-state levels of these, and of all, phospholipids detected in erg26-1 cells were found to be comparable to those seen in wild-type cells (Fig. 5, C versus D). Under the conditions of our assay, we did not detect measurable amounts of CDP-DG, phosphatidylglycerol or cardiolipin. Thus, we cannot say whether the erg26-1 mutation affects the rates of biosynthesis or steady-state levels of these lipids.

**TABLE II**

4α-Carboxysterol-C3 dehydrogenase activity in wild type and erg26–1 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>4α-Carboxysterol-C3 dehydrogenase relative activity</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
</tr>
<tr>
<td>erg26–1</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>erg26–1 pRS-ERG26</td>
<td>112 ± 4</td>
</tr>
</tbody>
</table>

FIG. 3. MS analysis of the GC peaks 6 and 7 found in temperature-shifted erg26-1 cells. Sterol extracts were obtained and diazomethanetreated as described in Fig. 2. MS, as described under “Experimental Procedures” was used to analyze all GC peaks, including peaks 6 and 7. A, peak 7, 4-methyl-4α-carboxy-diunsaturated sterol; B, peak 6, 4α-carboxy-diunsaturated sterol.

**FIG. 4.** erg26-1 cells have defects in neutral lipid metabolism. For pulse (A and B) and steady-state (C and D) labeling of neutral lipids, cells grown in synthetic media were shifted to 23 °C (A and C) or 37 °C (B and D) and incubated with [14C]acetate for 30 min (pulse) or 4 h (steady-state). Radiolabeled neutral lipids were extracted using chloroform, methanol (2:1) and analyzed by one-dimensional TLC using the solvent system hexane, ethyl ether, acetic acid (80:20:2). The percent lipid values are the average of five independent experiments. Abbreviations used are: TG, triacylglycerides; DG, diacylglycerides; MG, monoglycerides; FA, fatty acids; FAA, fatty acid alcohols; ERG, ergosterol; SE, steryl esters; **, sterol intermediates.

The Increased Accumulation of Toxic Sterol Intermediates Causes the Loss of Growth of erg26-1 Cells at the Nonpermissive Temperature—We explored the physiology underlying the ts defect in erg26-1 cells. Our studies focused on determining whether the loss of ergosterol biosynthesis or the increased accumulation of specific zymosterol intermediates was responsible for the loss of growth at the nonpermissive temperature.
To address this question, we examined whether feeding ergosterol or certain antifungal compounds to erg26-1 cells resulted in growth at high temperatures. The antifungal compounds chosen were those that targeted the sterol pathway, and would, or would not, reduce the accumulation of the zymosterol intermediates when fed to erg26-1 cells.

To perform the ergosterol feeding studies, we needed to genetically manipulate our erg26-1 strain. This is because, under normal aerobic growth conditions, yeast cells will not take up exogenous sterols from the media (1). However, they can be made to take up these lipids by overexpressing the gene, SUT1 (36). Thus, erg26-1 cells were transformed with the high copy plasmid pJR1133-SUT1. erg26-1 cells carrying this plasmid were then examined for their ability to grow at high temperatures in the absence and presence of ergosterol.

Our feeding studies revealed that ergosterol supplementation was unable to suppress the ts growth defect of our erg26-1 mutant strain. We found that erg26-1 cells incubated on plates containing 15 μg/ml ergosterol were able to grow at 25 °C but were unable to grow at the nonpermissive temperature for growth, 37 °C (Fig. 6A). The concentration of ergosterol we used has been shown to be sufficient for the growth of erg26Δ strains (17). We know that the Sut1p was functioning, because we were able to detect the uptake of radiolabeled ergosterol in all of the strains overexpressing SUT1. Moreover, we found that the rates of sterol internalization among the strains were nearly identical. Thus, the lack of ts suppression by ergosterol was not due to a reduced rate of uptake in erg26-1 cells.

In contrast, our antifungal feeding studies showed that certain antifungal compounds were able to suppress the ts growth defect of our erg26-1 mutant (Fig. 6B). Strikingly, the ability of these antifungal compounds to suppress this defect correlated with their ability to reduce the accumulation of the zymosterol intermediates (Fig. 6C). Terbinafine is an allylamine antifungal compound that targets the squalene epoxidase enzyme encoded for by the ERG9 gene (1). Erg9p function occurs upstream of Erg26p in the sterol pathway. We found that in the presence of 1.25 μg/ml terbinafine erg26-1 cells were able to grow at the nonpermissive growth temperature (Fig. 3B). We also found that the addition of this concentration of terbinafine caused a drastic reduction in the levels of the zymosterol intermediates in erg26-1 cells (Fig. 6C). We obtained identical results using fluconazole, which is anazole antifungal that targets the lanosterol C-14 demethylase encoded for by the ERG11 (1). Erg11p also functions upstream of Erg26p in the sterol pathway.

In contrast, the addition of 20 ng/ml fenpropimorph, a morpholine antifungal compound that targets the Erg2p sterol C-8 isomerase enzyme that is located downstream of Erg26p, did not suppress the ts growth defect of erg26-1 cells (Fig. 6B). Moreover, the addition of fenpropimorph did not cause a reduction in the accumulation of the zymosterol sterol intermediates (Fig. 6C). Thus, we conclude from our results that a reduction in the accumulation of the zymosterol intermediates in erg26-1 cells is required and responsible for any antifungal-dependent ts growth suppression. Therefore, our results strongly suggest that the accumulation of these toxic intermediates in erg26-1 cells causes cell death at high temperatures rather than the loss of ergosterol biosynthesis.

*Altering Sphingolipid Biosynthesis Does Not “Bypass” the*
Cell Lethality Associated with the Loss of Zymosterol Biosynthesis—SUR4/ELO3 and FEN1/GNS1/ELO2 encode for fatty acid elongase enzymes that are required for the biosynthesis of C26 fatty acids (37). C26 fatty acids are used exclusively to synthesize complex sphingolipids in yeast (38). Studies have demonstrated that mutations in SUR4 or FEN1 cause changes in the sphingolipid composition of cells (37). Interestingly, previous work also has shown that loss of function mutations in SUR4 or FEN1 can “bypass” the essentiality of ERG2 (23), whereas mutations in FEN1 allow strains lacking the ERG24 gene to survive (22). The reactions catalyzed by ERG24 and ERG2 lie just upstream and downstream of the ERG25-, ERG26-, and ERG27-catalyzed demethylation reactions, respectively (1). Thus, we wanted to determine whether altering the sphingolipid composition in strains lacking ERG25, ERG26, or ERG27 would allow for growth in the absence of zymosterol biosynthesis.

We tested for sphingolipid-dependent bypass of ERG25, ERG26, or ERG27 function using 5-FOA sensitivity assays. 5-Fluoroorotic acid is toxic to URA3 cells, but not ura3Δ cells (39). Plasmids carrying the URA3 gene are routinely negatively selected for by streaking cells onto plates containing 5-FOA. With this in mind, we constructed haploid strains that were deleted for ERG25, ERG26, or ERG27, and SUR4, and which harbored a URA3-containing centromeric plasmid (pRS414) carrying the respective deleted wild type sterol gene. We reasoned that, if altering sphingolipid biosynthesis in erg25Δ, erg26Δ, or erg27Δ cells through the loss of SUR4 bypasses the requirement for zymosterol biosynthesis, cells lacking each individual sterol gene and SUR4 would be able to deselect the URA3-containing plasmid and grow on plates containing 5-FOA. If, on the other hand, the loss of SUR4 does not suppress the requirement for proper zymosterol biosynthesis, cells would retain the plasmid and die on the 5-FOA plates.

As a control for sphingolipid-dependent suppression, we first attempted to demonstrate that loss of function of SUR4 allowed strains that lack ERG2 to live (23). However, we found that, in our W303-1A strain background, erg2Δ haploid cells were viable. There have been conflicting reports as to whether ERG2 actually is essential (23, 40). On the other hand, we were able to show, for the first time, that deleting SUR4 allowed strains that lacked ERG24 to grow (Fig. 7, A and B). Thus, loss of SUR4 function is able to suppress the strain-dependent essential requirement for ERG2, as well as the essential requirement for ERG24 function. In contrast, we found that deleting SUR4 in strains lacking ERG25, ERG26, or ERG27 did not bypass the essential requirement for zymosterol biosynthesis (Fig. 7, A and B). We obtained similar results when we examined whether the loss of FEN1 could suppress the need for proper zymosterol biosynthesis. Moreover, we found that deleting SUR4 in erg26-1 cells harboring pRS414-URA3-ERG26 did not allow for growth on 5-FOA plates at the nonpermissive temperature for erg26-1 cell growth (Fig. 7, C and D). Taken together, these results indicate that altering sphingolipid biosynthesis in erg25Δ and erg26Δ cells cannot suppress the cell toxicity associated with the accumulation of zymosterol intermediates, or the loss of cyclic sterol biosynthesis in erg27Δ cells.

Erg26p and Erg27p Localize to the Endoplasmic Reticulum, and Erg26p Physically Interacts with Erg25p—As a means of further understanding the physiology underlying the ts phenotype of erg26-1 cells, we determined the subcellular localization of Erg26p and Erg27p. Plasmids expressing galactose-inducible N-terminal GFP fusion proteins of Erg26p and Erg27p were transformed into wild type cells. Cells harboring these vector sequences were grown to exponential phase in raffinose-containing media, and subsequently shifted to galactose for 3 h to allow for the induction of fusion protein expression. The localization of GFP-Erg26p and GFP-Erg27p was then determined by fluorescence microscopy.

We found that in galactose-induced cells both GFP-Erg26p and GFP-Erg27p localized to a specific perinuclear organelle in yeast (Fig. 8, C and E). These localization patterns were not seen in raffinose-grown cells. Nuclear staining using the DNA-binding dye, 4′,6-diamidino-2-phenylindole, verified that the GFP fluorescence emitted by cells expressing these fusion proteins surrounded the nucleus and not the vacuolar membrane. Moreover, the visual localization of these fusion proteins looked identical to that of the ER-resident GFP-Sur4p (Fig. 8, A versus C and E). Thus, we conclude that both Erg26p and Erg27p reside within the ER in yeast.

Erg25p already has been localized to the ER (19). This protein contains a putative ER retention signal at its C terminus (41). Hydroxylation scans of the amino acid sequences of Erg26p and Erg27p do not reveal the presence of putative transmembrane domains or ER retention signals. Based on this information, we hypothesized that Erg26p and Erg27p may localize to the nucleus through their binding to Erg25p. We used the yeast two-hybrid assay as a means to test this hypothesis and determine whether Erg25p could bind Erg26p and/or Erg27p. The yeast two-hybrid assay uses protein-protein interaction-dependent transcriptional expression as a means to assay for protein complexation (42). Thus, we determined the up-regulation of β-galactosidase expression by assaying for activity in cells expressing various combinations of Erg proteins. The re-
results of the two-hybrid study are shown in Table III.

We found that we could detect a strong interaction between Erg25p (Table III, row, pACT2-ERG25) and both Erg26p (column, pBD-GAL4-ERG26; 127 ± 5.6 Miller units) and Erg27p (column, pBD-GAL4-ERG27; 85.2 ± 2.7 Miller units) but could not detect any interaction between Erg26p (row, pACT2-ERG26) and Erg27p (row, pACT2-ERG27) under the two different conditions tested (7.9 ± 3.3 and 4.8 ± 2.4 Miller units).

When the various ERG genes were expressed alone, the β-galactosidase activity levels were similar to the background value obtained from expressing both empty vectors together (column, pBD-GAL4 Cam).

Thus, the values that we obtained demonstrating the interactions between Erg25p and both Erg26p and Erg27p are not due to the self-activation of β-galactosidase expression by single ERG genes alone. We conclude from these data that Erg26p and Erg27p are most likely tethered to the ER through their association with the ER-resident Erg25p.

DISCUSSION

In a screen designed to isolate cells with defects in sphingolipid metabolism, we isolated erg26-1, a mutant strain that harbors a weakened allele of the ERG26 sterol gene. Based on the recent work of Bammert and Fostel (43), it seems reasonable to believe that sterol mutants would be isolated in screens aimed at isolating sphingolipid mutants. These investigators used microarray technology as a means to examine the effects of several drugs on genome wide transcription. Included in their study were azole compounds that targeted the sterol pathway. Using these drugs, they found that the levels of PS, PE, and PC in pulse-labeled erg26-1 cells were comparable to wild type cells grown at these same temperatures (Fig. 5). In temperature-shifted erg26-1 cells, the rate of biosynthesis of TG was decreased, whereas DG and MG synthesis was increased. The most straightforward explanation for these defects is that the accumulation of zymosterol intermediates block TG synthesis, thus, the accumulation of DG and MG. However, an alternative explanation is that one, or both, of the zymosterol intermediates are regulating at some level the individual activities required for TG, DG, and MG synthesis. Regardless of which hypothesis is true, the type of regulation exerted by these zymosterol intermediates, whether it is at the level of transcriptional or post-translation, remains to be resolved.

We also determined that the levels of ergosterol and sterol esters were decreased in erg26-1 cells. Defects were seen under all experimental conditions. The decrease in ergosterol most likely is an indirect effect of weakened 4α-CD activity rather than the regulation of the ERG4-encoded sterol C-24 reductase (1). If Erg4p activity was down-regulated, the accumulation of the ergosterol precursor ergosta-5,7,22,24(28)-tetraenol should have been detected in erg26-1 cells, because a sterol containing a C-24(28) double bond has been shown to accumulate in erg4 cells (47). The reduction in sterol ester levels may be a combined result of a reduction in ergosterol and the inability of the yeast acyl-CoA:cholesterol O-acyltransferase enzymes (48) to utilize the accumulated zymosterol intermediates as substrates.

The levels of PS, PE, and PC in pulse-labeled erg26-1 cells grown at either the permissive or nonpermissive temperatures were comparable to wild type cells grown at these same temperatures (Fig. 5, A versus B). Thus, the biosynthetic rate of phospholipid synthesis through the CDP-DG pathway (49) does not seem to be affected in erg26-1 cells. This would suggest that the levels of CDP-DG in erg26-1 cells and wild type cells are comparable. On the other hand, the rate of biosynthesis of PA (1.9-fold increase) was altered in temperature-shifted erg26-1 cells (Fig. 5, A versus B). How might PA accumulate in temperature-shifted erg26-1 cells? Quite possibly through lipid-dependent inhibition of the yeast PA phosphatase activities responsible for the conversion of PA to DG (50, 51). Lipid-dependent regulation of the 45- and 104-kDa PA phosphatase genes would have a dramatic impact on sphingolipid composition. Thus, there is precedent for the coordinate regulation of sterol and sphingolipid metabolism, at least at the level of transcription. Interestingly, at least one additional gene thought to be involved in regulating sterol metabolism has been isolated in the screen described in this study.2

By using TLC and GC/MS analyses, we have predicted that the zymosterol intermediates accumulating in erg26-1 cells are 4β-methyl-4α-carboxy-cholesta-8, 24-dien-3β-ol, and 4α-carboxy-cholesta-8,24–3β-ol. A previous study has shown that 4β-methyl-4α-carboxy-cholesta-8,24–3β-ol accumulates in cells lacking the ERG26 gene (17). This result was based on GC/MS analyses. The 4α-carboxy-cholesta-8,24–3β-ol species was not detected in these same cells. Based on the enzymatic mechanism for the demethylation of 4,4-dimethylzymosterol (46), 4α-carboxy-cholesta-8,24–3β-ol would be the second round carbonyxysterol substrate for C-3 sterol dehydrogenase. Thus, one would predict that 4α-carboxy-cholesta-8,24–3β-ol can only accumulate in cells possessing some degree of 4α-CD activity. This activity would have to be compromised to allow for 4α-carboxy-cholesta-8,24–3β-ol intermediate accumulation. This is borne out by our biochemical assays of 4α-CD activity in erg26-1 cells.

We found that erg26-1 cells harbored defects in neutral lipid synthesis (Fig. 4). In temperature-shifted erg26-1 cells, the rate of biosynthesis of TG was decreased, whereas DG and MG synthesis was increased. The most straightforward explanation for these defects is that the accumulation of zymosterol intermediates block TG synthesis, thus, the accumulation of DG and MG. However, an alternative explanation is that one, or both, of the zymosterol intermediates are regulating at some level the individual activities required for TG, DG, and MG synthesis. Regardless of which hypothesis is true, the type of regulation exerted by these zymosterol intermediates, whether it is at the level of transcriptional or post-translation, remains to be resolved.

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2 S. Mandala, personal communication.
activities has already been shown, because these enzymes are inhibited by the sphingoid bases dihydroxyinososine and phytosphingosine.\textsuperscript{3} Determining whether these long-chain bases accumulate in \textit{erg26-1} cells, and if so, whether they exert an effect on one or both PA phosphatase activities, is presently being studied in our laboratory.

In addition, we found that the rate of biosynthesis of PI was down 1.7-fold in temperature-shifted \textit{erg26-1} cells (Fig. 5, \textit{A versus B}). PI is synthesized from CDP-DG and inositol by PI synthase (53). A large body of work has pointed to intracellular inositol availability as the primary mode of regulation of PI synthase (54–56). Inositol is taken up from the media by the inositol permease encoded for by the \textit{ITR1} gene (57). Inositol uptake may be compromised in temperature-shifted \textit{erg26-1} cells due to the accumulation of zymosterol intermediates. The reduction in intracellular inositol due to zymosterol intermediate-dependent inhibition of \textit{Itr1p} activity or \textit{ITR1} expression would result in a decrease in PI levels. We must point out, that because CDP-DG levels were not determined in our studies, we cannot rule out that the decrease in PI levels we see in temperature-shifted \textit{erg26-1} cells is not through a reduction in CDP-DG levels.

We used fluorescence microscopy to localize GFP-Erg26p and GFP-Erg27p to the endoplasmic reticulum. When these fusion proteins were expressed in their respective haploid deletion cells, their localization was disrupted. This, together with the finding that the rate of biosynthesis of PI was down 1.7-fold, suggests that the sterols may play a role in the synthesis of PI.

\textbf{Acknowledgments}—We acknowledge A. Hoeft for performing the GC-MS analysis on microsomal reactions. We thank Drs. Chris Beh and Jasper Rine for various plasmids. We thank members of the Bill Bergman and Tom Edlind laboratories for many helpful discussions.

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The Effect of the erg26-1 Mutation on the Regulation of Lipid Metabolism in Saccharomyces cerevisiae


doi: 10.1074/jbc.M100274200 originally published online January 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100274200

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