ELO2 and ELO3, Homologues of the Saccharomyces cerevisiae ELO1 Gene, Function in Fatty Acid Elongation and Are Required for Sphingolipid Formation

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ELO2 and ELO3 were identified from the Saccharomyces cerevisiae genome database as homologues of ELO1, a gene involved in the elongation of the fatty acid 14:0 to 16:0. Mutations in these genes have previously been shown to produce pleiotropic effects involving a number of membrane functions. The simultaneous disruption of ELO2 and ELO3 has also been shown to produce synthetic lethality, indicating that they have related and/or overlapping functions. Gas chromatography and gas chromatography/mass spectroscopy analyses reveal that null mutations of ELO2 and ELO3 produce defects in the formation of very long chain fatty acids. Analysis of the null mutants indicates that these genes encode components of the membrane-bound fatty acid elongation systems that produce the 26-carbon very long chain fatty acids that are precursors for ceramide and sphingolipids. Elo2p appears to be involved in the elongation of fatty acids up to 24 carbons. It appears to have the highest affinity for substrates with chain lengths less than 22 carbons. Elo3p apparently has a broader substrate specificity and is essential for the conversion of 24-carbon acids to 26-carbon species. Disruption of either gene reduces cellular sphingolipid levels and results in the accumulation of the long chain base, phytosphingosine. Null mutations in ELO3 result in accumulation of labeled precursors into inositol phosphoceramide, with little labeling in the more complex mannolysated sphingolipids, whereas disruption of ELO2 results in reduced levels of all sphingolipids.

In the yeast Saccharomyces cerevisiae, sphingolipids comprise approximately 10% of the total membrane lipid species (1). The hydrophobic moiety of these lipids is ceramide, which consists of a long chain base coupled to a very long chain fatty acid that is almost exclusively 26:0 or hydroxy 26:0 (2). Although sphingolipids are relatively minor membrane lipid species, they are highly concentrated on the plasma membrane and appear to be essential for a number of critical membrane and cellular functions (3–5). Inhibition of sphingolipid biosynthesis results in growth inhibition and cell death (6, 7). Ceramide has also been implicated as a component of an essential cell signaling pathways in Saccharomyces (8).

In wild type cells, most fatty acids are 12–18-carbon species that are found in glycerolipids. Those species appear to be formed de novo by the well characterized soluble cytoplasmic fatty acid synthase complex. The very long chain (20+ carbon) fatty acids found in sphingolipids, however, are formed by membrane-bound fatty acid elongation systems that are not well characterized. These enzyme systems extend 14–18-carbon fatty acids by 2-carbon units by a sequence of reactions similar to those catalyzed by fatty acid synthases, with the exception of one reduction step, which in mammalian cells appears to be mediated by cytochrome b5 (9).

We recently identified a gene (ELO1) that encodes a membrane protein involved in the elongation of 14:0 to 16:0 (10). Comparison of the amino acid sequence of that gene with the recently completed Saccharomyces genome data base revealed two additional genes with high identity to ELO1. These are referred to as ELO2 and ELO3 in this paper, based on their function in fatty acid elongation. Both genes have been previously identified as open reading frames of unknown function; mutations in these genes induce pleiotropic phenotypes that appear to play a key role in membrane and cytoskeletal functions (11).

ELO2 was initially cloned by complementation of mutants of GNS1 (12). Those mutants confer resistance to echinocandins and have defects in β-glucan synthase activities. It was also reported as FEN1 (11, 13), a gene whose mutants exhibited bud localization defects and resistance to the sterol isomerase inhibitor SR 31747. ELO3 was previously cloned and identified, respectively, as APA1 with mutant alleles that cause a decrease in the level of the plasma membrane ATPase (14), SUR4 (15), whose mutants suppress the reduced viability on starvation mutant phenotype (rus161), and SRE1 (11) whose mutants suppress the effects of the sterol isomerase inhibitor, SR 31747. At least two laboratories have reported that simultaneous disruption of ELO2 and ELO3 produces a lethal phenotype (11, 13) which indicates that their encoded proteins have related and overlapping functions. Similar studies in this laboratory also support the synthetic lethality of the double disruptions.

The characterization of the genes described in this paper suggests that they encode proteins required for the production of very long chain fatty acids. Each gene apparently encodes a single enzyme component of one or more systems that elongate C16 and C18 acids to C20–C26 very long chain fatty acids. Disruption of either gene causes either the reduction or loss of 26:0, the end product of the elongation pathway, with a con-
Cells were washed 2 times with 5 ml of cold H2O and treated with 5% ELO3 polypeptide encoded by the ELO3 gene. Elo1p, polypeptide encoded by the ELO1 gene, was ligated into the KpnI/SalI site of YEps32, the HindIII site was removed by the self-ligation of the HindIII/PstI site. A 2.9-kb DNA fragment containing the HindIII site was digested with the HindIII/PstI site.

**Materials and Methods**

**Bacterial and Yeast Strains**—The strains used in this study and their genotypes are described in Table I. Plasmids constructed for this study are shown in Table II. Standard yeast genetic methods were used for construction of strains bearing the appropriate mutations (16). Cell growth conditions and growth medium have been previously described (16). Escherichia coli DH5α was obtained from Life Technologies, Inc. Saccharomyces cerevisiae cells were cultured as described previously (10).

**Lipid Analysis**—Fatty acid analysis of long and very long chain fatty acid methyl esters was performed by HCl-methanolysis of whole cell lipids as described previously (10). Methyl esters were prepared from washed cells grown to a density of 2–3 × 107 ml in 50 ml of CM or CM (–URA) medium containing either 2% glucose or 2% galactose. Gas chromatography temperature programming was modified to optimize analysis of very long chain fatty acids on a 0.32 mm × 30 m Supelco-Wax column.

**Sphingolipid Synthesis**—Two-ml cultures were grown to 2 × 109 cells/ml in CM medium at 30 °C and then labeled with either 20 μCi/ml [3H]serine for 6 h or 1 μCi/ml [3H]dihydrosphingosine (10 μM) for 30 min. Cultures were chilled on ice with an additional 0.5 ml of unlabeled trichloroacetic acid at 4 °C for 20 min. Lipids were extracted twice in 1 ml of chloroform/methanol/4.2 N NH4OH (9:7:2), and subjected to TLC as described (7). Radioactive bands were quantified on a Molecular Dynamics PhosphorImager using a tritium screen and subjected to MALDI mass spectrometry. The alkali-stable [3H]serine-labeled lipids were dried under N2, resuspended in 0.2 ml of chloroform/methanol/4.2 N NH4OH (9:7:2), and applied to silica gel TLC plates (25 μl/slot). After development, plates were dried in a fume hood and visualized by autoradiography. The radioactive bands were scraped off the plates and counted in a liquid scintillation counter.

**Construction of the ELO2 Δ Strain**—A 1.2-kb DNA fragment containing the coding sequence for ELO2 was derived by PCR using strain DTY10A genomic DNA as a template and primers ELO2A and ELO2B in Table III. The amplified product was ligated into the pCR-HIS cloning vector (Invitrogen), resulting in formation of pCRELO2. This plasmid was digested with HpaI/MfI to remove most part of the ELO2 coding region. A 1.2-kb DNA fragment of the Saccharomyces H3S3 gene was inserted into blunt-ended sites of the vector. The resulting plasmid (pCRELO2HIS) contains the H3S3 gene coding sequence in an orientation opposite to that of ELO2. A 2.2-kb elo2A::HIS3 linear disruption cassette was released from that plasmid by digestion with SalI and BclI.

This DNA fragment was electroporated into strain DTY10A to create strain DTY004. Interruption of the ELO2 gene by that fragment was confirmed by PCR, using primers that flank the ELO2 open reading frame sequence.

**Construction of the ELO3 Null Strain**—The plasmid pCRELO2 was digested with BamHI and SalI. The released 1.2-kb DNA fragment was ligated into plasmid YCpGAL1URA downstream of the GAL1 promoter sequences. This construct (YCpGALelo2U) was analyzed by diagnostic restriction enzyme digest to confirm the correct orientation of the ELO2 fragment in respect to the GAL1 promoter. That plasmid was electroporated into strain CSY3H for over-expression of the ELO2 gene in the elo2Δ background.

**Construction of the ELO3 Disruption Fragments**—A 2.9-kb DNA fragment containing the coding sequence for ELO3 was derived by PCR using primers ELO3A and ELO3B (Table III) and strain DTY10A genomic DNA as a template. The PCR product was digested with KpnI and SalI and ligated into plasmid YEps32 in which the HindIII site had been destroyed to produce YEpelo3. That plasmid was digested with HindIII followed by Klenow fill-in to remove the ELO2 coding region. A 1.2-kb DNA blunt-ended SalI/XhoI fragment containing the S. cerevisiae HIS3 gene was inserted into the vector creating plasmid YEpelo3HIS. The null, gene disrupted allele of ELO2; elo3Δ, a null, gene disrupted, allele of ELO3; GC/MS, gas chromatography/mass spectroscopy.

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**Table I**

| Saccharomyces cerevisiae strains used in this study |
|-----------------------------------------------|
| **DTY10A** | MATa, leu2-2,112,can1–100,ura-3–1,ade2–1, his3–11, his3–15 |
| **DTY003** | MATa, elo1Δ::HIS3,leu2-2,112,can1–100,ura-3–1,ade2–1, his3–11, his3–15 |
| **DTY004** | MATa, elo2Δ::HIS3,leu2-2,112,can1–100,ura-3–1,ade2–1, his3–11, his3–15 |
| **CSY3H** | MATa, elo3Δ::HIS3,leu2-2,112,can1–100,ura-3–1,ade2–1, his3–11, his3–15 |

**Table II**

| Plasmid | Description |
|---------|-------------|
| YCpGAL1URA | Derived from plasmid YCp50URA. Contains an 800-base pair fragment that includes the Saccharomyces GAL1 promoter ligated between the NruI and HindIII unique sites. |
| pCRELO2 | A 1.2-kb DNA fragment of the yeast ELO2 gene encompassing the entire protein coding sequence (from –76 to +1169) where the A of the ATG start codon is +1 and 1041 base pairs of an adjacent open reading frame, ligated into the SrfI site on pCRscript II-SK+ |
| YEpselo3 | A 2.9-kb DNA fragment containing approximately 1 kb of open reading frame of the yeast ELO3 gene, ligated into the KpnI/SalI site of YEps32, the HindIII site was removed by the self-ligation of the HindIII/PstI site |
| pCRElo2HIS | A 1.2-kb DNA fragment of the yeast HIS3 gene ligated into the MfeI/HpaI site of pCRELO2, created by deleting most of the ELO2 protein coding region |
| YEpelo3HIS | A 1.2-kb DNA fragment of the yeast HIS3 gene ligated into the HindIII site of YEpelo3 which removed most of the ELO3 protein coding region |
| YCpGAELO2(U) | A BamHI/SalI digestion of the pCRELO2 released 1.2-kb DNA fragment containing the protein coding sequence of the ELO2 gene. This was ligated into the BamHI/SalI sites on plasmid YCpGAL1 containing galactose-inducible GAL promoter and the URA3 gene as a selectable marker |
| YCpGALELO3(U) | Plasmid YCpGAL containing the ELO3 gene under control of the GAL1 promoter |

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**Table III**

| Gene | Oligonucleotide designation and sequence |
|------|----------------------------------------|
| ELO1–5’ | 5’-GCCAGTTAGTAAAGTTGGG-3’ |
| ELO1–3’ | 5’-GGACTGTTAATGTTGTGTTG-3’ |
| ELO2–5’ | 5’-GAAGCTTTTGCTCAGAAC-3’ |
| ELO2–3’ | 5’-GTCGAATATATATAAACAAATCCATCC-3’ |
| ELO3–5’ | 5’-GTCGAATACGCGTTCGGAG-3’ |
| ELO3–3’ | 5’-GTCCTGGATTATTCG-3’ |

2 The abbreviations used are: kb, kilobase pair(s); PCR, polymerase chain reaction; Elo1p, polypeptide encoded by the ELO1 gene; Elo2p, polypeptide encoded by the ELO2 gene; Elo3p, polypeptide encoded by the ELO3 gene; elo1Δ, a null, gene disrupted allele of ELO1; elo2Δ, a

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**Construction of the ELO2 Δ Strain**—A 1.2-kb DNA fragment containing the coding sequence for ELO2 was derived by PCR using strain DTY10A genomic DNA as a template and primers ELO2A and ELO2B in Table III. The amplified product was ligated into the pCR-HIS cloning vector (Invitrogen), resulting in formation of pCRELO2. This plasmid was digested with HpaI/MfI to remove most part of the ELO2 coding region. A 1.2-kb DNA fragment of the Saccharomyces H3S3 gene was inserted into blunt-ended sites of the vector. The resulting plasmid (pCRELO2HIS) contains the H3S3 gene coding sequence in an orientation opposite to that of ELO2. A 2.2-kb elo2A::HIS3 linear disruption cassette was released from that plasmid by digestion with SalI and XbaI. This DNA fragment was electroporated into strain DTY10A to create strain DTY004. Interruption of the ELO2 gene by that fragment was confirmed by PCR, using primers that flank the ELO2 open reading frame sequence.

**Construction of the ELO3 Null Strain**—The plasmid pCRELO2 was digested with BamHI and SalI. The released 1.2-kb DNA fragment was ligated into plasmid YCpGAL1URA downstream of the GAL1 promoter sequences. This construct (YCpGALelo2U) was analyzed by diagnostic restriction enzyme digest to confirm the correct orientation of the ELO2 fragment in respect to the GAL1 promoter. That plasmid was electroporated into strain CSY3H for over-expression studies of the ELO2 gene in the elo2Δ background.
resulting plasmid (YEpelo3HIS) contains the HIS3 gene coding sequence in an orientation opposite to that of ELO3. A 3.4-kb eloΔ::HIS3 linear disruption cassette was released from that plasmid by KpnI/SalI restriction digest. This DNA fragment was electroporated into strain KpnI linear disruption cassette was released from that plasmid by all three proteins.

Construction of GAL1-ELO3 Over-expression Strain—A DNA fragment containing the GAL1 promoter derived from vector YCpGAL1 by EcoRI digestion was inserted upstream of the ELO3 mRNA coding sequences in plasmid YEpelo3 at a SacI restriction site. The recovered fragment was ligated into complementary EcoRI digestion. The recovered fragment was ligated into complementary EcoRI sites on plasmid YCpGAL1 which contains the URA3 gene as a selectable marker. These constructs with an error rate of approximately 1 in 105 base pairs of DNA. All PCR reactions used either a 50 or 55 °C annealing temperature. Extension times at 72 °C were typically between 2.0 and 4.0 min or were adjusted to the size of the expected product. PCR reactions were routinely run for a total of 30 cycles. A list of all PCR primers used in this work appears in Table III.

Polymerase Chain Reaction—The polymerase chain reaction (PCR) was performed according to standard protocols (20). All PCR reactions were performed in a “OmniGene” thermal cycler (Hybaid, Ltd.) using a heat-stable recombinant “Vent” DNA polymerase with 5′- and 3′-exo nuclease activity. This allowed for greater fidelity of PCR products with an error rate of approximately 1 in 106 base pairs of DNA. All PCR reactions used either a 50 or 55 °C annealing temperature. Extension times at 72 °C were typically between 2.0 and 4.0 min or were adjusted to the size of the expected product. PCR reactions were routinely run for a total of 30 cycles. A list of all PCR primers used in this work appears in Table III.

RESULTS

Two genes were identified from the S. cerevisiae genome data base that had high identity to ELO1, a gene involved in the fatty acid synthase-independent elongation of 14:0 to 16:0 (10). ELO2 is located on yeast chromosome III at the locus designated YCR34W. ELO3 is located on chromosome XII at the locus designated YLR372W. Fig. 1 shows regions of homology between ELO1, ELO2, and ELO3 protein coding sequences. ELO2p and ELO3p are, respectively, 76 and 72% similar and 56 and 52% identical to that of Elo1p. The three genes contain multiple regions of contiguous identical residues throughout the protein sequence. Hydropathy analyses of ELO1p, ELO2p, and ELO3p by the Tmpredict algorithm (Fig. 2) suggest that the three proteins contain five membrane-spanning regions. The identified core regions of these sequences align identically with previously predicted transmembrane regions of Elo1p (10). The regions of highest identity in all three genes lie between presumptive transmembrane-spanning regions II and III (Fig. 2). That region has 16 identical amino acid residues located between residues 185 and 200 of Elo3p (from the amino terminus) and contains a cluster of four consecutive basic residues followed by an HXXHH motif, which has been previously identified with fatty acid desaturase, ribonucleotide reductase, hemerithrin, and other iron-containing proteins (9). ELO2p also has a hydrophobic stretch in that region that contains several polar residues, suggesting.
that it might serve as a hydrophobic cleft associated with an active site of the enzyme. The amino- and carboxyl-terminal regions of Elo2p and Elo3p are most dissimilar to Elo1p. In all three proteins the C-terminal sequences that follow transmembrane segments contain unusually high numbers of basic residues, suggesting that these domains have some homologous function. Both Elo2p and Elo3p peptide sequences lack a GSA motif near the carboxyl terminus of Elo1p, which is proposed to be an NADPH binding site for a number of lipid biosynthetic enzymes (10).

Disruption of Elo2 or Elo3 Produces Changes in Long Chain Fatty Acid Chain Length Distribution—Quantitative gas chromatography and GC/MS analysis of fatty acid methyl ester fractions indicate that the 26:0 and HO-26:0 species are absent in the elo2Δ strain. Sharply reduced levels of 26:0 (approximately 20% wild type levels) and HO-26:0 (approximately 40% wild type levels) were found in the elo2Δ strain (Fig. 3, Table V). The reductions in C26 species were accompanied by increases in minor (and in some cases, undetectable) wild type species. Lipids from the elo2Δ strain displayed new peaks with retention times between 9 and 11 min. GC/MS analysis indicated that they were hydroxy 16- and 18-carbon fatty acids with fragmentation patterns consistent with the ω-hydroxy fatty acid standards. (In wild type, those species were not detected.) No C20 fatty acids were detected in the elo2Δ strain and the total C20–C26 species were 30% of wild type levels.

The elo3Δ strain contained elevated levels of C20 and C22 fatty acids (Table V). The most abundant species was 22:0 which averaged 3.1% of the total fatty acyl mass, a 10-fold increase over wild type levels. Large increases in the levels of hydroxy C16–C24 fatty acids were also observed in this strain. Unlike the elo2Δ strain, Elo3 disruption resulted in an approximate 20% increase in the total levels of very long chain species.

Over-expression of Elo2 or Elo3 Alters Fatty Acid Composition of, but Does Not Compensate for, the Complementary Gene Disruption—The synthetic lethality of elo2Δ and elo3Δ suggests that their encoded proteins have overlapping functions. To test whether increased activity of Elo2p and Elo3p can compensate for loss of the other’s function, their genes were each placed under the control of the strong, inducible GAL1 promoter and transformed into cells with the disrupted homologue. The very long chain fatty acid distributions of these strains and wild type are shown in Table V and Fig. 4.

Very long chain fatty acid distributions of wild type, elo2Δ, and elo3Δ controls (which did not contain the plasmid) on the non-fermentable carbon source, galactose, were similar to those found for these same strains when grown on glucose medium (Table V). Both gene-disrupted strains contained 16- and 18-carbon-hydroxylated species. The elo2Δ strain exhibited reduced levels of 26:0 and HO-26:0 and undetectable levels of C20 and C22 species, whereas the elo3Δ strain showed the characteristic large increase in C20 and C22 fatty acids and no C26
species. Galactose-induced over-expression of Elo3p in an elo2Δ/GAL1-ELO3 strain resulted in a 50-fold decrease in the hydroxy 16:0 fatty acids and reduced levels of the less abundant hydroxy 16:1, 18:0, and 18:1 species (Table V). The strain also had slight, but significant, increases in hydroxy 26:0 (which is elevated to wild type levels) and 26:0 (which is elevated from 15 to 36% of the wild type levels).

More dramatic changes were observed on over-expression of Elo2p in the elo3Δ/GAL1-ELO2 strain. This failed to restore the missing 26-carbon species and also did not reduce the characteristic high levels of 22:0. Large increases in C20 and C24 carbon species were observed, however. There was a 44-fold increase in 24:0 and a 5-fold increase in hydroxy 24:0 (Table V, Fig. 4). Similar increases were observed in 20:1 (2-fold) and 22:1 (5-fold). Induction of Elo2p in this strain also produced high levels of a 24:1 species that was not detected in the elo3Δ or wild type strains. All of the shorter chain hydroxy 16:0, 16:1, 18:0, and 18:1 species present in the elo3Δ strain were reduced to undetectable levels in cells containing over-expressed Elo2p.

Sphingolipid Synthesis in Elo2Δ and Elo3Δ Strains—The effects of disrupting ELO1, ELO2, and ELO3 on sphingolipid biosynthesis are shown in Fig. 5, A and B. The strains were pulse-labeled (30 min) with [3H]dihydrosphingosine (lanes 1–5) or labeled for 6 h with [3H]serine (lanes 6–9), and the lipid extracts were chromatographed by TLC either before or after mild alkaline methanolysis, respectively. The methanolysis deacylates fatty acids on phospholipids that are linked to the glycerol-head group moiety by O-acyl bonds, but leaves the N-acyl ester bonds of sphingolipids intact. A thin layer chromatogram of those lipids is shown in Fig. 5A. The lipids in wild type and elo1Δ cells had a similar pattern; most of the label incorporated into sphingolipids was found in the major inositol-containing species including inositol phosphorylceramide and mannosyl diinositoldiphospho-

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**TABLE IV**

Distribution of 14–18 carbon-saturated and -unsaturated fatty acids in wild type, elo2Δ, and elo3Δ strains

These species represent >95% of the total fatty acids in wild type cells. Values are indicated as weight % of total fatty acid methyl esters.

| Strain     | 14:0a | 14:1 | 16:0 | 16:1 | 18:0 | 18:1 |
|------------|-------|------|------|------|------|------|
| Wild type  | 2.2 ± 0.3 | 0.8 ± 0.0 | 15.0 ± 1.1 | 41.8 ± 1.9 | 5.4 ± 1.3 | 34.8 ± 0.5 |
| elo2Δ      | 1.2 ± 0.5 | 0.3 ± 0.1 | 16.7 ± 1.0 | 40.2 ± 4.0 | 8.2 ± 2.3 | 33.4 ± 2.1 |
| elo3Δ      | 2.5 ± 0.2 | 1.5 ± 0.5 | 15.7 ± 3.7 | 49.7 ± 4.6 | 4.8 ± 0.7 | 25.8 ± 1.0 |

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*a* Fatty acids are designated according to standard nomenclature in which the first number designates the number of carbon atoms in the hydrocarbon chain. The number following the colon indicates the number of double bonds in the acyl chain.

*b* Fatty acid levels are expressed as weight % of total 14–16 carbon fatty acid species.

*c* ±S.D. of at least three independent experiments performed on 3 different days.
ELO2 and ELO3 Function in Fatty Acid Elongation

The roles of Elo2p and Elo3p as components of the very long chain fatty acid elongation system are supported by several observations. Hydrophobicity analysis indicates that Elo2p and Elo3p are intrinsic membrane proteins with multiple membrane-spanning regions, which is consistent with the reported tight membrane association of fatty acid elongation activities (9). ELO2 and ELO3 encode polypeptides that have a high degree of homology to the ELO1 gene product which is involved in the highly specific elongation of 14 carbon fatty acids (10). Disruption of either gene alters the composition of very long chain fatty acids and causes the accumulation of intermediate length fatty acid precursors.

The lethality caused by the simultaneous disruption of the ELO2 and ELO3 genes indicates that their products have a high degree of overlapping functions. The different pattern of accumulation of very long chain fatty acid intermediates in the elo2Δ and elo3Δ strains, however, suggests that Elo2p and Elo3p may play roles in independent, and to some degree parallel, metabolic pathways. These may be located in different parts of the cell. Previous studies of fatty acid elongation enzyme activities in Saccharomyces suggest, in fact, that there

Quantitation of the [3H]dihydrosphingosine labeling by PhosphorImager is shown in Fig. 5B. The majority of the converted label (81%) was found in the sphingolipid fraction in the wild type and elo1Δ strains. In these lipids, approximately 90% of the label was in the form of inositol phosphosphingosine and mannosyl phosphoryceramide and 10% was in the form of mannosyl diinositoldiphosphoryceramide. Approximately 2% of the converted dihydrosphingosine label was in phytosphingosine, 10% in ceramide, and 8% found in phospholipid fractions. The label in phospholipids is derived from the degradation of dihydrosphingosine to an aldehyde intermediate which is then converted to a fatty acid. In elo2Δ and elo3Δ, the amount of converted dihydrosphingosine label was reduced to approximately 30% of wild type due to reductions in ceramide and sphingolipids. Phytosphingosine was increased approximately 3-fold in the elo2Δ strain and by 1.5-fold in elo3Δ, whereas the label incorporated into the phospholipid fractions was similar to that observed in wild type. A similar pattern of dihydrosphingosine labeling was found in wild type cells that were treated with the ceramide synthase inhibitor, australifungin, as seen in Fig. 5A. These data suggest that disruption of ELO2 or ELO3 has the effect of reducing the level of ceramide synthesis which results in the concomitant reduction in cellular sphingolipid levels.

DISCUSSION

TABLE V

Distribution of very long chain fatty acids and hydroxy fatty acids in wild type, elo2Δ::HIS3 and elo3Δ::HIS3 strains

| Strain          | 16:0-         | 18:0-         | 18:1-         | 20:0-         | 20:1-         | 22:0-         | 22:1-         | 24:1-         | 24:0-         | 26:0-         |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|                 | (glucose)     | (galactose)   | (galactose)   | (glucose)     | (galactose)   | (galactose)   | (galactose)   | (galactose)   | (galactose)   | (galactose)   |
| Wild type       | 0.11 ± 0.02   | 0.17 ± 0.03   | 0.16 ± 0.03   | 0.12 ± 0.02   | 0.14 ± 0.03   | 0.12 ± 0.02   | 0.14 ± 0.03   | 0.14 ± 0.03   | 0.12 ± 0.02   | 0.14 ± 0.03   |
| elo1Δ           | 0.09 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.11 ± 0.02   | 0.15 ± 0.03   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.16 ± 0.03   | 0.13 ± 0.02   | 0.16 ± 0.03   |
| elo2Δ           | 0.08 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.10 ± 0.02   | 0.14 ± 0.03   | 0.14 ± 0.02   | 0.15 ± 0.03   | 0.16 ± 0.03   | 0.12 ± 0.02   | 0.14 ± 0.03   |
| elo3Δ           | 0.08 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.10 ± 0.02   | 0.14 ± 0.03   | 0.14 ± 0.02   | 0.15 ± 0.03   | 0.16 ± 0.03   | 0.12 ± 0.02   | 0.14 ± 0.03   |
| elo1Δ elo2Δ     | 0.08 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.11 ± 0.02   | 0.15 ± 0.03   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.16 ± 0.03   | 0.13 ± 0.02   | 0.16 ± 0.03   |
| elo1Δ elo3Δ     | 0.08 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.11 ± 0.02   | 0.15 ± 0.03   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.16 ± 0.03   | 0.13 ± 0.02   | 0.16 ± 0.03   |
| elo2Δ elo3Δ     | 0.08 ± 0.01   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.11 ± 0.02   | 0.15 ± 0.03   | 0.15 ± 0.02   | 0.17 ± 0.03   | 0.16 ± 0.03   | 0.13 ± 0.02   | 0.16 ± 0.03   |

Values indicated are weight % of total cellular fatty acid methyl esters ± S.D. for at least three independent experiments. Fatty acyl species were identified by GC mass spectroscopy as described under "Materials and Methods."
Clues about the enzymatic characteristics of Elo2p can be seen from the analysis of the \( ELO2^{1} \), elo3\( ^D \) strain. The absence of a \( \mathrm{C}26 \) fatty acid indicates that Elo2p cannot catalyze the elongation of \( 24:0 \) to \( 26:0 \). Elo2p apparently has the highest catalytic specificity for \( \mathrm{C}20 \) acyl-CoA, which results in the observed accumulation of \( 22:0 \). Experiments in which \( ELO2 \) is over-expressed in elo3\( ^D \) cells indicate that Elo2p can convert \( 22:0 \) to \( 24:0 \) with less efficiency. This reduced activity toward \( \mathrm{C}22 \) substrates can be compensated for by over-expression of Elo2p which shifts in the accumulated species from \( 22:0 \) to \( 24:0 \).

Elo3p appears to act on a broader range of substrates. Twenty-six-carbon fatty acids are formed when the masking Elo2p activity is removed in the \( ELO3^{1} \), elo2\( ^D \) strain. The absence of \( \mathrm{C}26 \) species in the elo3\( ^D \) strain, together with the absence of \( \mathrm{C}26 \) species in the elo3\( ^D \) strain, indicates that conversion of \( 24:0 \) to \( 26:0 \) is exclusively performed by the Elo3p-dependent elongation system. This might result in the inability of ceramide synthase to make contact with available substrates.

The marked changes in cellular sphingolipid fatty acid composition in elo2\( ^D \) and elo3\( ^D \) strains provide a rational explanation for the pleiotropic effects reported for mutant alleles of these genes. Mutations or disruptions of \( ELO2 \) and \( ELO3 \) were previously reported as affecting \( \beta \)-glucan synthase activity (12), the plasma membrane, (\( \mathrm{H}^+ \))-ATPase (14), bud localization defects (13), and resistance to sterol synthesis inhibitors (11, 13). All of these functions are apparently produced by the activities of intrinsic membrane proteins, which could be affected by changes in the composition of their lipid environment or by the absence of an essential sphingolipid-membrane protein interaction.

Although Elo2p and Elo3p appear to be essential for the formation of very long chain fatty acids, there are apparently a number of other components that catalyze other steps in the elongation cycle that have yet to be identified. The lack of information about the structure, function, and cellular location of these enzymes gives an unclear picture of how they are organized and how intermediates are transferred from one
reaction center to the next. Until now, the intrinsic hydrophobicity of these enzymes has hindered previous attempts to purify and characterize the components of these systems. The identification of ELO2 and ELO3 in this paper provides new tools that can be used to resolve questions concerning the mechanism, organization, and structure of these complex systems.

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**FIG. 5.** A, TLC autoradiograph of lipids extracted from strains labeled with [3H]dihydrosphingosine and [3H]serine. Total lipid extracts from [3H]dihydrosphingoine-labeled cells (lanes 1–5) or [3H]serine-labeled lipids subjected to mild alkaline hydrolysis (lanes 6–9) were prepared from wild type (lanes 2 and 6), elo1Δ (lanes 3 and 7), elo2Δ (lanes 4 and 8), or elo3Δ (lanes 5 and 9) gene-disrupted strains. Lane 1 is wild type strain treated with 25 ng/ml australifungin for 15 min prior to [3H]dihydrosphingosine addition. Lipids were identified with authentic standards: CER, ceramide; DHS, dihydrosphingosine; PE, phosphatidylethanolamine; PHS, phytosphingosine; PC, phosphatidylcholine; IPC, inositol phosphoceramide; MIPC, mannosylinositol phosphoceramide; M(IP)2C, mannosyl diinositolphosphorylceramide; GPE, glycerophosphoethanolamine; GPS, glycerophosphoserine; GPC, glycerophosphocholine. B, levels of dihydrosphingosine-labeled lipids in wild type (open bars), elo1Δ (diagonal lines), elo2Δ (horizontal lines), and elo3Δ (cross-hatched lines) strains. [H]-Labeled lipids from TLC fractionation similar to that shown in A were quantified by PhosphorImaging using a Molecular Dynamics tritium phosphor screen. Relative levels of emission units ± S.D. are shown for lipids fractionated in three independent experiments as described under “Materials and Methods.”
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