Ceramide/Long-Chain Base Phosphate Rheostat in Saccharomyces cerevisiae: Regulation of Ceramide Synthesis by Elo3p and Cka2p

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Sphingolipid precursors, namely, ceramide and long-chain base phosphates (LCBPs), are important growth regulators with often opposite effects on mammalian cells. A set of enzymes that regulate the levels of these precursors, referred to as a ceramide/LCBP rheostat, is conserved in all eukaryotes. In order to gain further insight into the function of the rheostat in Saccharomyces cerevisiae, we searched for mutants that are synthetically lethal with a deletion of the LCB3 gene encoding LCBP phosphatase. In addition to acquiring expected mutants lacking the LCBP lyase, the screen revealed elo3 (sur4) mutants that were defective in fatty acid elongation and cka2 mutants lacking the α′ subunit of the protein kinase CK2 (casein kinase). Both mutations affected the in vivo activity of the acyl coenzyme A (acyl-CoA)-dependent fumonisin B1-sensitive ceramide synthase (CS). The Elo3 protein is necessary for synthesis of C26-CoA, which in wild-type yeast is a source of C26 fatty acyls found in the ceramide moieties of all sphingolipids. In the in vitro assay, CS had a strong preference for acyl-CoAs containing longer acyl chains. This finding suggests that a block in the formation of C26-CoA in yeast may cause a reduction in the conversion of LCBs into ceramides and lead to an overaccumulation of LCBPs that is lethal in strains lacking the Lcb3 phosphatase. In fact, elo3 mutants were found to accumulate high levels of LCBs and LCBPs. The cka2 mutants, on the other hand, exhibited only 25 to 30% of the in vitro CS activity found in wild-type membranes, indicating that the α′ subunit of CK2 kinase is necessary for full activation of CS. The cka2 mutants also accumulated high levels of LCBs and had elevated levels of LCBPs. In addition, both the elo3 and cka2 mutants showed increased sensitivity to the CS inhibitors australifungin and fumonisin B1. Together, our data demonstrate that the levels of LCBPs in yeast are regulated by the rate of ceramide synthesis, which depends on CK2 kinase activity and is also strongly affected by the supply of C26-CoA. This is the first evidence indicating the involvement of protein kinase in the regulation of de novo sphingolipid synthesis in any organism.

Cellular membranes are complex structures whose lipid compositions change depending on their subcellular localization, extracellular signals, and environmental conditions. Mechanisms of biogenesis and maintenance of these dynamic structures and the role of individual lipid species in cell growth and survival are not fully understood and have been a subject of increased interest in recent years. Saccharomyces cerevisiae, nearly all of whose genes involved in lipid metabolism have been identified (many of them in recent years [6]), provides a convenient model for such studies. It has long been recognized that yeast cells can tolerate the complete lack of some membrane lipids, e.g., phosphatidylycerine (1), but that proper concentrations of others are absolutely required for viability, e.g., sterols. In fact, the sensitivity of fungi to membrane sterol imbalance is the mechanism underlying most successful antifungal agents (10).

Sphingolipids, similar to sterols, are important constituent membrane lipids that are critical for the normal function and viability of both yeast and mammalian cells. These essential molecules comprise a diverse group of ceramide-containing lipids that are predominantly localized in the outer leaflets of the plasma membranes of eukaryotic cells, where they play important structural roles in cell recognition and adhesion (14) and in the formation of functional lipid microdomains (44). In addition, short-lived sphingolipid precursors, namely, long-chain bases (LCBs), long-chain base phosphates (LCBPs), and ceramides (N-acyl-LCBs), have been shown to affect cell growth, differentiation, and death (16). Ceramides play prominent roles in stress responses and programmed cell death (15, 21) and are often viewed as growth-inhibitory mediators. In contrast, the major mammalian LCBP sphingosine-1-phosphate has been shown to protect cells from ceramide-mediated apoptosis (5) and has been implicated as a growth-promoting second messenger (46). It has been proposed that the dynamic balance between ceramide and sphingosine-1-phosphate is important for determining cell fate (47). This balance is maintained by a group of sphingolipid metabolic enzymes, which can be referred to as the ceramide/LCBP rheostat (42, 47).

The ceramide/LCBP rheostat is conserved in yeast, despite the fact that there are significant differences between the major yeast and mammalian sphingolipids. For example, the inositol-phosphorylceramides (IPCs), mannosyl-IPCs, and inositol-phosphoryl-mannosyl-IPCs, the only sphingolipids found in yeast, are not present in mammalian cells (7). However, yeast genes encoding enzymes that are involved in the metabolism of sphingolipid precursors, including LCB kinase, LCBP phosphatase, LCBP lyase, and ceramidase, and recently discovered genes operating ceramide synthesis, LAC1 and LAG1, were all found to have homologues in mammalian cells (12, 19, 28, 29, 21) and are often viewed as growth-inhibitory mediators. In contrast, the major mammalian LCBP sphingosine-1-phosphate has been shown to protect cells from ceramide-mediated apoptosis (5) and has been implicated as a growth-promoting second messenger (46). It has been proposed that the dynamic balance between ceramide and sphingosine-1-phosphate is important for determining cell fate (47). This balance is maintained by a group of sphingolipid metabolic enzymes, which can be referred to as the ceramide/LCBP rheostat (42, 47).

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Changes in the levels of LCBPs and ceramides also accompany stress responses and affect cell growth in yeast. Mild heat stress causes transient increases in LCBs and LCBPs and a sustained increase in free ceramides (8, 17, 45). Mutant cells that accumulate moderate levels of LCBPs are more resistant to heat stress (29, 31, 45); however, the overaccumulation of LCBPs (up to a 400-fold increase over the basal level) observed in mutants defective in both LCBP phosphatase and LCBP lyase is lethal (18, 48). As with phosphates, the levels of free ceramide have to be carefully regulated. Free-ceramide levels in cells that are defective in the IPC synthase or treated with the selective IPC synthase inhibitor aureobasidin A are approximately eightfold increased subsequent to cell death (35). It is possible that an increase in free ceramide plays a role in cell death because cells that are defective in ceramide formation can survive a blockage of IPC synthase activity (35, 43). In order to gain further insight into the function of the ceramide/LCBP rheostat in yeast, we set up a genetic screen for mutants that require the LCB3 (Ysr2/Lbp1 acyl coenzyme A (acyl-CoA)-dependent ceramide synthase.

**MATERIALS AND METHODS**

**Strains, media, and general growth conditions.** The *S. cerevisiae* strains used in this study are listed in Table 1. The screening strain Y388-2 was obtained from strain Y388 (2) by PCR-based deletion of the LCB3 gene. Briefly, a G418 resistance marker was PCR amplified from plasmid DNA (13) and homologous recombination was directed by incorporating 40 bp of complementing genomic sequences at the 5′ ends of the primers. Standard yeast genetic procedures were used to obtain and verify gene deletions (13). Strain Y388-DD was obtained by deletion of the *DPL1* gene in strain Y388-2. The *DPL1* gene was amplified from *S. cerevisiae* genomic DNA, digested by the complementing colored-phenotype method.

**TABLE 1. Genotypes of strains used in this study**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| Y388  | MATa ade2 ade3 ura3 leu2 lys2 | 2 |
| Y382  | MATa ade2 ade3 ura3 trp1 | This study |
| Y388-2 | MATa lcb3::KAN ade2 ade3 ura3 leu2 lys2 | This study |
| Y382-2 | MATa lcb3::KAN ade2 ade3 ura3 leu2 trp1 | This study |
| Y388-DD | MATa lcb3::KAN dpl1::LEU2 ade2 ura3 leu2 lys2 | This study |
| Y388-2p | MATa lcb3::KAN ade2 ade3 ura3 leu2 lys2 [URA3 LCB3 ADE3] | This study |
| MSS204 | MATa leu3 mel1 trp1 his4 dpl1::LEU2 | 45 |
| BY4741 | MATa his3 leu2 met15 ura3 | Research Genetics |
| BY4742 | MATa his3 leu2 his2 ura3 | Research Genetics |
| BY4741el02 | MATa elo2::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741el03 | MATa elo3::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741aka2 | MATa cka2::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741lkcl | MATa cka1::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741ckb1 | MATa ckb1::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741ckb2 | MATa ckb2::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741dpl1 | MATa dpl1::KAN his3 leu2 met15 ura3 | Research Genetics |
| BY4741el03 | MATa elo3::KAN his3 leu2 lys2 ura3 | Research Genetics |

Australafungin fermentation and isolation. Australafungin was purified from *Sporormiella australis* ATCC 74157 according to the methods of Mandala et al. (27). Briefly, *S. australis* was grown on solid cracked-corn-based fermentation medium and extracted after 14 days of fermentation with ethyl acetate. Australafungin was purified by use of both gel column and high-speed countercurrent chromatography. Purity was approximately 90%.

**Plasmid construction.** The screening plasmid L3A3-1 (*LCB3 ADE3 URA3*) was constructed from a pRS416 backbone containing the *URA3* marker and a *CEN* replicon. *LCB3* was subcloned from the genomic library plasmid pJAB15-1 (40) and inserted between the *Nor1* and SacI restriction sites of similarly digested pRS416. The *ADE3* gene was amplified from *S. cerevisiae* genomic DNA, digested with *Smal* and *SacII*, and cloned into the *LCB3*-containing plasmid. The integrity of the *LCB3* gene was confirmed by sequencing, and *ADE3* was confirmed by the complementing colored-phenotype method.

**Synthetic-lethality screen and colony-sectoring assay.** The colony-sectoring assay is based on colony color selection with adenine mutants. *S. cerevisiae ade2* strains accumulate a red pigment, whereas *ade2 ade3* strains do not and phenotypically result in a normal white colony. The colony-sectoring assay was performed by transforming the Y388-2 screening strain with plasmid L3A3-1. The resulting strain, Y388-2p, was grown under selective pressure in SC−ura in order to retain the plasmid. The screening strain Y388-2p is functionally ade2 and should appear as a red colony. However, frequent plasmid loss in yeast occurs at the approximate rate of 1% per cell per generation and thus results in a colony with a red- and white-sectored phenotype when it is grown under nonselective conditions (i.e., on YEPD agar).

**Agr diffusion assay.** Agr diffusion was used to evaluate sensitivity of the screening strain to ceramide synthase inhibitors and other antifungals and to identify antifungal sensitivity differences among the synthetically lethal mutants.

30, 32, 33, 36, 41, 43, 49). Changes in the levels of LCBPs and ceramides also accompany stress responses and affect cell growth in yeast. Mild heat stress causes transient increases in LCBs and LCBPs and a sustained increase in free ceramides (8, 17, 45).
For this assay, cells grown in SC–ura were washed one time in sterile phosphate-buffered saline. Cells (10^7) were added to 4 ml of 0.8% agarose in YEPD and plated on 10-cm-diameter petri dishes. Compounds were spotted on the preincubated agar plates at the indicated volumes and concentrations. Growth and color development were monitored after 4 days.

Mutant complementation with a genomic library. Mutants E37, U48, U4, and U33 were transformed with a yeast genomic library based on the pS66 CEN vector (ATCC 77162) by selecting for leucine prototrophs. The transformants (20,000 to 50,000) were pooled and plated on SC plates containing 1 mg of 5-FOA per ml. Plasmid DNA was recovered by E. coli transformation from 5-FOA-resistant clones that formed white colonies and retransformed into respective mutants. Genomic DNA inserts in plasmids that conferred colony sectoring and a 5-FOA-resistant phenotype were identified by sequencing both ends of the insert DNA and matching the sequence with the S. cerevisiae genome sequence (4). Plasmids containing the ELO3 (SUR4) gene were recovered by complementing mutants E37 and U48, but only plasmids carrying the LCB3 gene were obtained in complementation experiments involving mutants U4 and U33. To obtain plasmids complementing mutants U4 and U33, pools of transformants were plated on SC plates containing 20 μM fumonisin B1 and prepared with agarose in place of agar. Plasmids pELO3 and pELO2 were used to test the ability of individual genes to complement the phenotype of synthetic-lethality mutants. These plasmids were obtained by ligase of pRS415 vector DNA (Stratagene) into BamHI and PstI digestion sites, respectively, and cut with the appropriate restriction enzymes to release the plasmid inserts. Genomic DNA inserts present on library plasmids to complement mutants U4 and U33, in vivo plasmid insert was verified by DNA sequencing. To test the ability of individual genes present on library plasmids to complement mutants U4 and U33, in vivo recombination in yeast cells was utilized to construct plasmids (26). Briefly, each gene was amplified by using primers containing 80 nucleotide sequences complementary to regions flanking the BamHI site in vector p366 and the mutant cells were cotransformed with the PCR product and BamHI-linearized vector to select for Leu1 transformants.

Determination of the MIC. Susceptibilities of various strains to ceramide synthase inhibitors were measured by using the broth microdilution method. For australifungin, cells were inoculated at an initial density of 10^5 cells/ml in a 96-well plate containing 200 μl of YEPD medium per well. Drug was added from a 10 mM stock solution in dimethyl sulfoxide. For fumonisin B1 (Sigma) experiments, 95 μl of SC inoculated with 10^7 cells/ml was mixed with 5 μl of an appropriate fumonisin B1 dilution prepared from a 10 mM stock solution in dimethyl sulfoxide. For fumonisin B1, cells were incubated for 30 min at 30°C with gentle shaking, and MICs were determined by counting cell number in triplicate after 7 days of incubation. Phospholipid membrane preparations. For this assay, cells grown in SC–ura were washed once in water and were incubated with an SC mixture (1:1). The mixture was centrifuged at 1,000 g for 5 min, and 100 μl of the stopped-reaction mixture along with 10 nmol of cold lignoceroyl-DH (Sigma) were incubated at 30°C for 2 h to room temperature. Phospholipids were degraded by the addition of 15 μl of 1.5 N KOH in methanol and a 30-min incubation at 37°C. Samples were neutralized with 15 μl of 1.74 N acetic acid in methanol. For LC analysis, 10-μl sample of AQC-reacted extract was resolved on HPLC TLC plates (Whatman) in solvent I (chloroform-methanol [19:1.5]) by using aQc derivatized phytosphingosine (PHS) and DHS as standards. For LC/MS analysis, 50 μl of AQC-reacted extracts was loaded on 500 mg C18 columns (Varian) that had been precolumned with 1 ml of a methanol-water mixture (1:1). The column was washed with 1.65 ml of methanol-water (1:1), and lipids were eluted with 1.5 ml of an acetonitrile-water mixture (8:1). Samples were dried under a stream of nitrogen, resuspended in 20 μl of methanol-water (9:1), and developed on LHPKD plates with solvent II (chloroform-methanol-1.26 N ammonium [76:34:6], with AQC-derivatized DH-1-phosphate (DHS-1-P) and PHS-1-P being used as standards. Fluorescently tagged lipids were visualized by UV light.

RESULTS

Genetic link between ceramide and LCBPs. LCBs formed in the endoplasmic reticulum can be used for synthesis of both ceramide and LCBPs (Fig. 1). Both genetic and biochemical evidence indicates that the overaccumulation of LCBPs is lethal in yeast cells (18, 45, 48). Under normal growth conditions, inactivation of either the LCBP phosphatase or the LCBP lyase (i.e., deletion of the LCB3 or DPL1 gene, respectively) has no effect on yeast cell growth. However, mutants with both genes deleted or dpl1 mutants challenged with high concentrations of sphingosine (41) are unable to grow. In both cases, viability is restored by an additional deletion of the major LCB kinase encoded by the LCB4 gene (18, 36, 48). These observations indicate that scenarios leading to gross accumulation of LCBPs are lethal and that LCB phosphates rather than free LCBs are responsible for cell death in these situations. Additionally, mutants defective in the LCB3 gene and therefore lacking major LCBP phosphatase are supersensitive to the ceramide synthase inhibitor australifungin (29), thus indicating that decreased consumption of LCBs for ceramide formation is reflected by the increased synthesis of LCBPs. In order to further explore this apparent association between the Lcb3 phosphatase and ceramide synthesis, we undertook a search for mutants that require an intact LCB3 gene for growth. First, we constructed a strain that would allow the application of a classic colony-sectoring assay (2) to screen for mutants synthetically lethal with an LCB3 gene deletion. The LCB3 gene was deleted from the chromosome of strain Y388, and the resulting strain, Y388-2, was confirmed to have an elevated steady-state level of long-chain phosphates similar to that of previously described lcb3 mutants (data not shown; 18, 45). The Y388-2 strain, which carries additional chromosomal
mutations in the ade2, ade3, ura3, and leu2 genes, was transformed with the centromere-based plasmid containing intact copies of the ADE3, URA3, and LCB3 genes. The cells in this strain form red colonies when they contain the plasmid and white colonies when they lack the plasmid. Since deletion of the LCB3 gene does not affect growth on YPD medium, the strain undergoes high-frequency plasmid loss under nonselective conditions and forms sectored red-white or white colonies (Fig. 2). We validated the utility of this strain for identification of mutants that are lethal in the lcb3 background by showing that the introduction of a dpl1 chromosomal deletion in the screening strain causes the expected LCB3 synthetic lethality and thus forms solid-red colonies on YEPD plates. Furthermore, we found that in an agar diffusion format, cells of the screening strain treated with the ceramide synthase inhibitor australifungin formed a distinct ring of red colonies surrounding the zone of the growth inhibition. This effect was selective for australifungin and was not caused by antifungal compounds with unrelated modes of action (Fig. 3). Similar effects were observed in experiments when cells were treated with various concentrations of drugs in a broth microdilution format of the growth assay. Cells in wells containing subinhibitory concentrations of australifungin and fumonisin B1 had a distinct red color, whereas the absence of drug or the presence of subinhibitory concentrations of cycloheximide, voriconazole, and amphotericin B did not result in a red color. Cells exposed to sublethal concentrations of ceramide synthase inhibitors turn red because they are forced to retain the plasmid that provides a functional Lcb3 phosphatase, thus indicating that the inhibition of ceramide synthesis leads to an elevation of LCBP above the level that can be tolerated by an lcb3 mutant. This result provides for the direct phenotypic manifestation of a genetic

FIG. 1. Metabolism of sphingoid LCBs in S. cerevisiae. The LCBs DHS and PHS are either N acylated by ceramide synthase or phosphorylated by Lcb4 (major) and Lcb5 (minor) kinases. LCBPs can be hydrolyzed by Dpl1 lyase, forming ethanolamine-1-phosphate and palmitaldehyde, or they can be dephosphorylated by Lcb3 (DHS-1-P selective) and Ysr3 (PHS-1-P selective) lipid phosphatases. C26-CoA, a preferred substrate for ceramide synthase, is formed during the elongation cycle that requires the ELO3 gene. Ceramide synthase activity is sensitive to the natural product inhibitors australifungin and fumonisin B1. As demonstrated by the results presented here, the activity of the α’ isoform of CK2 kinase encoded by CK42 is necessary for the development of full ceramide synthase activity. MIPC, mannosyl-IPC; M(IP)2C, inositolphosphoryl-mannosyl-IPC.

FIG. 2. Schematic representation of the results of a Δlcb3 synthetic-lethality screen. A Δlcb3 screening strain constructed for the purpose of the synthetic-lethality screen forms red colonies when it contains the LCB3 plasmid and white colonies when it lacks the plasmid. Under nonselective growth conditions, this strain undergoes frequent plasmid loss, resulting in formation of sectored red and white colonies. Mutations that give advantage to cells that retain the plasmid will result in solid-red colonies.

1. Screening strain: ade2 ade3 lcb3 ura3 leu2
2. Screening plasmid: ADE3 LCB3 URA3
3. Chromosomal mutation lethal with Δlcb3
4. Chromosomal mutation complemented by LEU2 plasmid from library

Colony Phenotype

- White
- Red/White
- Red
- Red/White

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link between ceramide synthesis and LCBP levels. As expected, the Y388-2p strain not only was an indicator of drugs that inhibit ceramide synthesis but also turned red upon being treated with sphingosine, DHS, and to a lesser extent PHS and an IPC synthase inhibitor, aureobasidin A (data not shown).

Identification of mutants synthetically lethal with a deletion of \( lcb3 \).

Cells of the screening strain were mutagenized by either UV or EMS treatment and plated on YEPD plates, and red colonies were selected for further analysis. One hundred fifty-eight red colonies were found in the pool of 200,000 colonies that survived mutagenesis. Thirty-six of these had a stable color phenotype and were sensitive to 5-FOA, which is consistent with their inability to lose the \( URA3 \) plasmid. Fourteen mutants changed their color phenotype from red to either red-white or white upon transformation with the \( LEU2 \) plasmid carrying the \( LCB3 \) gene. These transformants also exhibited 5-FOA resistance. The purpose of this plasmid-shuffling experiment was to eliminate mutants whose red color phenotype and 5-FOA sensitivity resulted from either gene conversion or plasmid integration events. Ten out of the remaining 14 \( \Delta lcb3 \) synthetically lethal mutants were ascribed to result from mutations in the \( DPL1 \) gene due to the reversion of the phenotype upon transformation with a \( LEU2 \)- and \( DPL1 \)-containing plasmid. The four remaining mutants were subjected to further analysis. All four mutants were found to carry recessive mutations based on the reversal of the red colony phenotype in the diploid strain obtained by mating the mutant with the Y382-2 strain.

Non-DPL1 mutants are hypersensitive to australifungin. The growth-inhibitory activity of the ceramide synthase inhibitor australifungin is potentiated by a deletion of the \( LCB3 \) gene, which likely results in the toxic accumulation of LCBP species (29). One possible outcome of the synthetic-lethality screen was to obtain mutations leading to a deficiency in ceramide formation. Such mutants would potentially be more susceptible to australifungin. To address this possibility, we tested the MICs of australifungin for the non-DPL1 mutants E37, U48, U4, and U33 (Fig. 4). All four mutants were supersensitive to the drug. However, the mutants split into two distinct susceptibility patterns. E37 and U48 were approximately 200-fold more sensitive to australifungin than the parent strain, whereas U4 and U33 showed a 30-fold increase in sensitivity. U9 and U63 are \( DPL1 \) mutants and showed only slight differences from the control (Fig. 4). None of the mutants displayed a substantial increase in sensitivity to amphoterocin B or aureobasidin A (data not shown).

ELO3 (SUR4) and CKA2 are required for normal growth of \( LCB3 \) deletion mutants. In order to identify mutations that resulted in \( LCB3 \) plasmid retention and increased sensitivity to australifungin in the four mutants, we transformed them with a yeast genomic library and assayed for a red colony color phenotype reversal. This approach was successful for mutants E37 and U48. Sequence analysis of plasmids extracted from independent clones revealed that both E37 and U48 were complemented by library plasmids that carry \( ELO3 \) (\( SUR4 \)), a gene which is responsible for the formation of \( C_{26} \) very-long-chain fatty acids (VLCFA) in yeast (37). In order to confirm that \( ELO3 \) and not one of the adjacent genes present in the library plasmid complemented the phenotype of E37 and U48...
mutants, we constructed a plasmid (pELO3) that carries the wild-type ELO3 gene on the single-copy vector and showed a reversal in colony color phenotype in the E37 and U48 mutants. This plasmid was unable to reverse the colony color phenotype of the U4 and U33 mutants. A similar plasmid that carried the ELO2 gene was unable to complement any of the four mutants. Furthermore, the ELO3 locus was amplified from each mutant and sequence analysis of two independent amplicons obtained from each strain revealed the presence of nonsense mutations in codons 236 and 200 of the E37 and U48 alleles, respectively, and the wild-type ELO3 sequence in mutants U4 and U33. These results indicate that, in strain Y388, inactivation of both the LCB3 and ELO3 genes results in a loss of viability.

Genes defective in mutants U4 and U33 were identified by complementation of the drug supersensitivity phenotype. In the complementation experiments, the ceramide synthase inhibitor fumonisin B1 was used in place of australifungin. For each mutant, a complementing plasmid carried a genomic DNA insert that contained the entire CKA2 gene encoding the α′ catalytic subunit of CK2 (casein) kinase. A plasmid containing a CKA2 gene not only reversed a fumonisin B1 supersensitivity phenotype but also caused a change in colony color from red to a sectored red-white in both the U4 and U33 mutants. Sequence analysis of the CKA2 locus amplified from both mutants revealed the presence of a nonsense mutation in codon 227 of mutant U4 and a frameshift mutation in codon 80 of mutant U33.

In order to verify that both the lcb3 elo3 double deletion and the lcb3 cka2 double deletion cause growth defects, we analyzed genetic crosses among deletion strains BY4742lcb3, BY4741elo3, and BY4741cka2. Twenty-one out of 23 spores that came from 5 nonparental ditype and 13 tetratype tetrads and that were deduced to be double lcb3 cka2 mutants formed distinctly small colonies. In the case of elo3 mutants, 14 out of 16 double-deletion spores either formed extremely small colonies (11 spores) or were inviable (3 spores). This result clearly shows that a strong negative synthetic interaction exists between the lcb3 and elo3 as well as the lcb3 and cka2 deletions; however, in most cases, the interaction results in a poor-growth phenotype rather than lethality.

Crude membranes from cka2 mutants have reduced ceramide synthase activity. In order to further characterize the nature of australifungin and fumonisin B1 sensitivity, we directly measured the activity of ceramide synthase in crude membrane preparations obtained from elo3 and cka2 mutants and the parental strains. Ceramide formation in this assay was linear with time (Fig. 5A), was dependent on the addition of long-chain acyl-CoA, and was completely inhibited by 100 μM fumonisin B1 and 10 μM australifungin (Fig. 5B). In a separate experiment, we determined that fumonisin B1 was a submicromolar inhibitor of yeast ceramide synthase activity (50% inhibitory concentration, 0.2 μM). We also found that the rate of the reaction increased with increasing lengths of acyl chains in the acyl-CoA substrate; e.g., there was fivefold more product formed in reaction mixtures that contained C24:0-CoA than in those that contained C16:0-CoA (Fig. 5C). Ceramide was not formed when free long-chain fatty acid was added to the reaction mixture, thus indicating that the measured activity was due to the acyl-CoA-dependent ceramide synthase and not due.
to the reverse activity of ceramidase (30). The specific activity of acyl-CoA-dependent ceramide synthase in the crude membrane preparations of the four non-DPL1 mutants obtained in the synthetic-lethality screen was consistently reduced compared to that of the parental strain Y-388. The activity in membranes from elo3 (E37 and U48) mutants was reduced by 30 to 40% when C20:0-CoA was used as a substrate. This difference, however, was significantly diminished when C24:0-CoA was used as a substrate in the assay (Fig. 6). Similar to the E37 and U48 elo3 mutants, the ELO3 deletion strain also had a slightly reduced ability to incorporate C20:0-CoA into ceramide in the in vitro enzyme assay. This defect was not present when C24:0-CoA instead of C20:0-CoA was used in the assay (Fig. 6).

The activity in membranes from cka2 mutants (U4 and U33) was reproducibly reduced by 70 to 75% regardless of the chain length of the acyl-CoA used in the assay (Fig. 6). The independently obtained cka2 deletion strain also had severely reduced ceramide synthase activity (Fig. 6).

The elo3 and cka2 mutants have elevated levels of LCBs and LCBPs. As mentioned previously, the combination of lcb3 and dpl1, two mutations that individually result in modest elevation of LCBP levels, leads to a large accumulation of LCBPs and results in cell death (18, 45, 48). We expected that other mutations that cause poor growth in the lcb3 deletion background would also have elevated levels of LCBPs. We measured the levels of both free LCBs and free LCBPs in four mutants obtained in the screen as well as in the Δcka2, Δelo3, and Δdpl1 deletion strains and the respective parental strains (Fig. 7). Both the elo3 mutants E37 and U48 and the elo3 deletion strain accumulated high levels of free DHS and PHS and also had highly elevated levels of LCBPs compared to those of parental wild-type strains. Similarly, the cka2 mutants U4 and U33 and the Δeka2 strain had highly elevated levels of PHS and elevated levels of DHS and LCBPs. The Δelo3 and Δeka2 mutants, like the Δdpl1 strain, accumulated high levels of LCBPs, but each of the mutants appeared to accumulate different species of LCBPs. Both the Δelo3 and Δcka2 mutants, consistent with the block in ceramide synthesis, accumulated both LCBS and LCBPs, whereas the Δdpl1 mutant, which lacks LCBP lyase, accumulated mostly LCBS.

Deletion of elo3 and deletion of cka2 result in hypersensitivity to ceramide synthase inhibitors. As shown above, the elo3 and cka2 mutants obtained in the genetic screen were supersensitive to drugs inhibiting acyl-CoA-dependent ceramide synthase. In order to confirm this conclusion, we measured the elo3 deletion and cka2 deletion strains for sensitivity to both australifungin and fumonisin B1. Similarly to the truncated elo3 mutants E37 and U48, an elo3 deletion strain, BY4741elo3, was about 200-fold more sensitive to australifungin than the parental strain, BY4741 (Fig. 8). Interestingly, the isogenic elo2 deletion strain was also more sensitive to the drug than the parent; however, it was much less sensitive than the elo3 mutant. Likewise, the deletion of the LCB3 gene had an observable but comparatively smaller effect on australifungin sensitivity than did the deletion of ELO3. The elo3 deletion mutant was also supersensitive to the structurally unrelated inhibitor of the ceramide synthase fumonisin B1, and as in the case of australifungin, a deletion of ELO3 sensitized the cells to fumonisin B1 much more so than a deletion of ELO2. The Δelcb3 strain was also sensitive to fumonisin B1. A Δeka2 strain similar to the cka2 mutants U4 and U33 also showed highly increased sensitivity to australifungin and fumonisin B1 (Fig. 8). Notably, mutants lacking other subunits of the CK2 kinase, namely, the Δcka1, Δkbb1, and Δkbb2 mutants, did not show any increase in sensitivity to fumonisin B1 (data not shown).
DISCUSSION

We have isolated and characterized \textit{S. cerevisiae} mutants that require the normally dispensable \textit{LCB3} gene for growth. Since \textit{Lcb3} LCB phosphatase is a part of the system that maintains LCBP-ceramide homeostasis, we expected to uncover mutations in this screen that affect other parts of this system. Out of 14 mutants identified in the screen and confirmed to carry mutations causing synthetic lethality with the \textit{LCB3} deletion, 10 were defective in the \textit{DPL1} gene encoding the LCBP lyase, 2 were mutated in the \textit{ELO3 (SUR4)} elongase gene required for formation of the C26 VLCFA, and the remaining 2 were defective in in vitro acyl-CoA-dependent ceramide synthase activity. Mutants defective in the ceramide synthase activity lacked a functional \textit{CKA2} gene encoding the $\alpha'$ catalytic subunit of the CK2 kinase, thus indicating that CK2 kinase is a novel determinant of a fully functional ceramide synthase. Deletion of \textit{DPL1} was previously demonstrated to be lethal in strains lacking \textit{Lcb3} phosphatase (18, 48). A single \textit{LCB3} or \textit{DPL1} deletion results in a 5- to 40-fold increase of the steady-state levels of the LCBPs and does not have a measurable effect on cell growth. However, elimination of both the Lcb3 phosphatase and the Dpl1 lyase activities from the cell causes massive accumulation of LCBPs, over 400-fold above the wild-type levels, which in turn leads to cell death. Recent detailed analysis of LCBP accumulation in yeast indicates that DHS-1-P species are more toxic than PHS-1-Ps and that the phosphatase rather than the lyase is involved in the removal of the toxic excess of these intermediates (48). Over

![Figure 7](image_url)  
FIG. 7. The $\Delta lcb3$ synthetically lethal mutants accumulate LCBs and LCBPs. LCBs and LCBPs were extracted from 12 OD units of \textit{Y388} background cells and from 30 OD units of \textit{BY4741} background cells, derivatized with AQC, and treated with KOH to deacylate glycerophospholipids as described in Materials and Methods. (A) Ten microliters of each extract was resolved by TLC in solvent I under conditions separating AQC-LCBs. (B) Fifty microliters of each extract was absorbed on a C18 column, and AQC-LCBPs that eluted from the column were separated by TLC in solvent II. The migration of AQC-derivatized standards is indicated. TLC plates were visualized with UV light. w-t, wild type.

![Figure 8](image_url)  
FIG. 8. Null \textit{elo3} and \textit{cka2} mutants are supersensitive to ceramide synthase inhibitors. Cells of various deletion mutants were exposed to australifungin (A) or fumonisin B$_1$ (B) in the broth microdilution assay, and the growth was measured after 48 h of incubation. WT, wild type.
two-thirds of the mutants in our screen were classified in the expected DPL1 category, indicating that the screen was reasonably saturated.

Mutants E37 and U48 carry mutations in the ELO3 gene, and their dependence for growth on the LCB3 plasmid was relieved by transformation with an ELO3 plasmid. Additionally, we confirmed by genetic crosses that spores carrying deletions of both the LCB3 and ELO3 genes have poor viability. elo3 mutants are unable to synthesize C_{26} VLCFA, which are the primary fatty acids linked by the yeast ceramide synthase to PHS or DHS to form the ceramide found in all yeast sphingolipids (7, 37) (Fig. 1). Deletion of ELO3 results in the incorporation of shorter-chain fatty acyls into ceramides, mostly C_{22} and C_{24} species that are formed with participation of the Eto2 protein. Additionally, ∆elo3 mutants appear to accumulate even shorter fatty acyl ceramides that are not found in wild-type cells (20, 37). ELO3 deletion mutants grow almost normally under standard laboratory conditions, but were shown to have elevated LCB levels (20, 37) (Fig. 8). These observations, together with our findings that elo3 mutations are lethal in cells lacking Lcb3 phospatase and that elo3 cells accumulate high levels of LCBPs, indicate that a defect in the formation of the C_{26} VLCFA in yeast causes a backlog in the flux through the sphingolipid pathway at ceramide synthesis, which in turn leads to the elevation of free LCB levels and increased production of the LCBPs. This scenario indicates a significant in vivo preference of the ceramide synthase for the C_{26}CoA substrate. This view is supported by our observation that the in vitro specific activity of ceramide synthase is nearly 40% higher in assays utilizing C_{24:0} than in assays utilizing C_{20:0}-CoA as a substrate (Fig. 5c). An even more dramatic preference for C_{26:0}-CoA was observed in the in vitro ceramide synthase assays by Guillas and coworkers (12). Mutants E37 and U48, as well as the elo3 deletion mutants, show extraordinary growth sensitivity to the ceramide synthase inhibitors australifungin and fumonisin B_{1} (Fig. 4 and 8). Since elo3 mutants have normal levels of in vitro acyl-CoA-dependent ceramide synthase activity, it is not likely that the lack of the Elo3 protein directly impacts the sensitivity of the enzyme to drugs. This sensitivity is more likely caused by the decreased consumption of LCBs in cells lacking C_{26}-CoA and the consequent elevation of LCBP levels, which must be elevated even further in cells treated with normally subinhibitory levels of ceramide synthase inhibitors. Interestingly, elo3 mutants were more sensitive to both fumonisin B_{1} and australifungin than were the elo2 and lcb3 mutants, which were also more susceptible to both drugs than was the homogenic wild-type strain (Fig. 8). It is possible that in the absence of C_{26}-CoA, a preferred substrate for ceramide synthase, the enzyme becomes significantly more sensitive to both drugs. Taken together, these results highlight the importance of the ELO3 gene and C_{26}-CoA formation for LCB homeostasis in yeast. This is likely a reflection of the extraordinary significance of VLCFA in the formation of functional plasma membranes in single-celled eukaryotes. Most of the C_{26} VLCFA in yeast are found in sphingolipids, which are located primarily in the plasma membrane (38). Yeast SLC mutants that are able to survive without making any sphingolipids produce compensatory glycerolipids that are based on a C_{26} VLCFA-containing phosphatidylinositol not found in normal cells (24), further stressing the importance of C_{26} VLCFA for the survival of yeast cells. Mutants that survive despite their inability to form ceram ide also produce novel glycerolipids, and indeed, preliminary experiments showed that they do contain VLCFA (12, 43). The plasma membrane lipid bilayer in yeast, as indicated by the lengths of predicted transmembrane domains in the membrane proteins, is 50% thicker than the lipid bilayers of internal membranes (25). It is possible that VLCFA-containing lipids are indispensable for thickening of the plasma membrane and therefore may be essential for protein sorting and for formation of functional cell envelope proteins in yeast.

The strain constructed for the purpose of our LCB3 genetic screen formed a distinct ring of red colonies surrounding the zone of inhibition due to australifungin treatment, which confirmed that the effect of a block in ceramide formation is exacerbated in lcb3 mutants (Fig. 3). This finding indicated that this genetic screen was capable of yielding mutants defective in ceramide synthase. We assayed acyl-CoA-dependent ceramide synthase activity in crude membranes isolated from four non-dpl1 mutants and found that specific enzyme activity was reduced three- to fourfold in mutants U4 and U33 (Fig. 6). Both mutants were also more sensitive to growth inhibition by australifungin and fumonisin B_{1} (Fig. 4), and both accumulated free LCBs and had increased levels of LCBPs (Fig. 7). Surprisingly, both mutants carried mutations inactivating the α’ catalytic subunit of the CK2 kinase encoded by the CKA2 gene. An independently obtained deletion of the CKA2 gene led to all phenotypes found in the U4 and U33 mutants, including a defect in in vitro ceramide synthase activity (Fig. 6 to 8). CK2 kinase, formerly known as casein kinase, is a highly conserved, ubiquitous, and pleiotropic protein kinase whose activity is essential for growth in yeast. The enzyme exists in cells as a heterotetrameric holoenzyme consisting of two catalytic subunits, α and α’, and two regulatory subunits, β and β’. A recent study demonstrated differential distributions of the holoenzyme and individual CK2 subunits in mammalian cells and showed that individual CK2 subunits are located on the cytosolic side of the rough endoplasmic reticulum and the smooth endoplasmic reticulum-Golgi complex (9). CK2 kinase has been implicated in numerous fundamental cell processes, including cell proliferation, regulation of polymerase III activity, cell polarity, ion homeostasis, signal transduction, and information storage in neuronal cells, although the cellular function of this kinase is still unclear (3, 11, 39). Our observations that the α’ subunit of CK2 kinase is selectively needed for optimal activity of ceramide synthase and that CKA2 mutants accumulate ceramide synthase substrates define a new important cellular role for CK2 kinase in the activation of ceramide synthase activity. This is the first example of regulation of sphingolipid synthesis in any organism (34). At this point, it is not known if CK2 kinase is directly involved in the activation of ceramide synthase through phosphorylation of the enzyme or if the kinase is required in some indirect way for the formation of the fully active enzyme.

The specific activities of ceramide synthase in membranes from the elo3 mutants E37 and U48, as well as from the elo3 deletion strain, were reduced in comparison to that of wild-type membranes when C_{20}-CoA was provided as a substrate. However, the ability to incorporate longer C_{24} fatty acyl-CoA into ceramide was not impaired in elo3 membranes (Fig. 6).
One possible explanation for this observation is that the acyl-CoA in our crude membrane assay may be further elongated in vitro. Thus, given the preference of the enzyme for longer-chain fatty acyl moieties, elo3 membranes that are less efficient in the elongation of the added substrate would make less ceramide.

All of the mutants isolated in the LC8B screen were affected in elements of the ceramide/LCBP rheostat itself. The fact that we did not find mutants defective in either the LAG1 or LAC1 determinants of the ceramide synthase activity is not surprising given the likelihood that these genes are highly redundant and that single mutants are not defective in the rate of ceramide synthesis. It is worth noting that we did not find mutations affecting genes that encode potential sensors or effectors of altered levels of LC8s or LCBPs, that is, mutations that themselves do not cause elevation of sphingolipid metabolites but rather result in increased sensitivity to the intracellular accumulation of LC8s. It is possible that such hypothetical effectors have subtle effects on cell growth and therefore would not be expected to emerge from our screen. One other possibility is that these lipids do not have highly selective receptors in yeast and exert their effects through localized changes in the physical properties of membrane bilayers rather than acting as sensu stricto secondary messengers.

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