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The *LCB2* gene of *Saccharomyces* and the related *LCB1* gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis

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ABSTRACT **The first and committed step in synthesis of the ceramide moiety of sphingolipids is catalyzed by serine palmitoyltransferase (EC 2.3.1.50), which condenses palmitoyl-CoA and serine to form 3-ketosphinganine. This step is thought to be tightly regulated to control the synthesis of sphingolipids, but data supporting this hypothesis are lacking mainly because the enzyme has resisted purification and consequent characterization. Rather than attempting to purify the enzyme from normal cells, we have taken a different tack and opted to try and overproduce the enzyme to facilitate its purification. Here we demonstrate that overproduction in *Saccharomyces cerevisiae* requires expression of *LCB1*, a previously isolated yeast gene, and *LCB2*, the isolation and characterization of which we describe. Several lines of evidence argue that both genes encode subunits of the enzyme; however, biochemical evidence will be needed to substantiate this hypothesis. Although overproduction was modest, 2- to 4-fold, it should now be possible to devise improved overproduction vectors for yeast or other host organisms.**

Sphingolipids are abundant components of the plasma membrane of eukaryotic cells. In animals, sphingolipids are known to play roles in general membrane function, cell-to-cell recognition, regulation of cell growth, differentiation, oncogenesis, and immune functions (for review, see refs. 1 and 2). Recent interesting results implicate sphingolipid breakdown products, sphingosine, sphingosine-1-phosphate, lysosphingolipids, and ceramide, as second messengers in incompletely characterized signal transduction pathways (3–8). An important implication of these data is that the concentration of sphingosine, ceramide, and related compounds, in addition to sphingolipids, must be tightly controlled. Little is known about the control mechanisms that operate on either the biosynthetic or degradative pathways.

The first and committed step in *de novo* sphingolipid synthesis is catalyzed by serine palmitoyltransferase [SPT, 3-ketosphinganine synthetase (EC 2.3.1.50), for review, see ref. 9], which condenses serine and palmitoyl-CoA to form a C-18 carbon unit, 3-ketosphinganine. Further reactions convert 3-ketosphinganine to the long-chain base sphingosine in animals and phytosphingosine in fungi and plants. *De novo* synthesis of sphingolipids in animals has been hypothesized to be controlled at the SPT step. Experiments showing that treatment of neurons with a long-chain base decreases SPT activity support this hypothesis (10). Further support for this hypothesis is lacking because essential experimental reagents—purified SPT, antibodies against the enzyme, and the SPT gene—are not available. SPT activity could be controlled by transcriptional or by posttranscriptional processes or by both in neuronal cells.

To begin to prepare the reagents necessary for understanding how *de novo* sphingolipid synthesis is regulated, we have utilized the genetically tractable organism *Saccharomyces cerevisiae*. Our goal was to isolate the SPT gene(s) to overproduce the enzyme, which, to our knowledge, has never been purified from any organism. To isolate the gene, we began with a mutant strain lacking SPT activity and requiring exogenous long-chain base for growth (Lcb$^-$) (11). Complementation of the Lcb$^-$ phenotype using a genomic DNA library resulted in the isolation of a gene, *LCB1* (12), whose predicted protein showed amino acid similarity to enzymes known to catalyze a reaction chemically similar to that catalyzed by SPT and to use the same cofactor (pyridoxal P) as SPT. These data suggested that the Lcb1 protein was the SPT enzyme or a subunit of the enzyme. During the course of these experiments a second complementation group, *lcb2*, with an Lcb$^-$ phenotype, was isolated and shown to be necessary for SPT activity (13). In this report we describe the isolation and characterization of the *LCB2* gene* and demonstrate that increased SPT activity is obtained only if both *LCB* genes are overexpressed.

EXPERIMENTAL PROCEDURES

Strains and Media. *S. cerevisiae* strains used in these experiments are presented in Table 1. Media were prepared as described (12). The YPH strains are sensitive to the long-chain base phytosphingosine (Sigma), and to transform these strains with plasmid DNA, it was necessary to use 6–12 μM phytosphingosine in 0.025% Tergitol [less than half the normal concentrations (12)] in selection plates. Likewise, for genetic crosses involving YPH strains, it was necessary to make the same adjustments in the composition of plates used for dissection of tetrads and growth of spores; otherwise, the spores did not germinate.

Plasmids. Plasmids were constructed using standard molecular cloning techniques. Insert B7 (Fig. 1) is a 7-kb *Bam*HI *S. cerevisiae* DNA fragment cloned into the *Bam*HI site of pRS315 (15). Insert B7ΔS (Fig. 1) is a 4.9-kb *Bam*HI–*Sal* I fragment cloned into pRS315 at the *Bam*HI–*Sal* I sites in the polylinker. Insert 2.3 (Fig. 1) is a 2.3-kb *Bam*HI–*Sac* I fragment cloned into pRS316 (15) at the *Bam*HI–*Sac* I sites of the polylinker. Insert LCB2-R (Fig. 1) is a 4.3-kb *Eco*RI fragment, made blunt-ended by using the Klenow fragment of DNA polymerase I, and ligated into pRS315 cut with *Sma* I. A deletion allele, *lcb2-Δ4*, was constructed using pRS315 carrying the 4.3-kb *Eco*RI fragment, by deleting the coding region between the *Cla* I and *Xba* I (Fig. 1) sites, filling-in the ends with the Klenow fragment of DNA polymerase I, and ligating to a 1.4-kb *TRP1ARS1* fragment. The *lcb2-Δ3* dele-

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Abbreviation: SPT, serine palmitoyltransferase.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M95669 for *LCB2*).

Table 1. Genotypes and origin of yeast strains used in these studies

Strain	Genotype	Ref.
BS238	*MATa lcb2 ura3-52 leu2-3, 112 ade1*	13
LCB25	YPH250 carrying the *lcb2-Δ4* allele	This work
LCB27	YPH250 carrying the *lcb1-Δ3* allele	This work
GRF123	*MATα trp1 ade1 rrp1 lys1 gal1*	14
YPH102	*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} his3-Δ200 leu2-Δ1*	15
YPH250	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ1 his3-Δ200 leu2-Δ1*	15
YPH500	*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	15
YPH501	*MATa/α ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	15

tion allele was constructed in the same way except that the 1.1-kb *URA3* DNA fragment was inserted into the gene. The *lcb1-Δ3* deletion allele was made by replacing the coding region (12) between the *Sal* I and *Bam*HI sites, which were made blunt-ended, with a 1.4-kb *TRP1ARS1* fragment. The integrating vector pRSLCB2-2 was constructed by cloning a 2.6-kb *Bam*HI–*Apa* I fragment, carrying the 5′ coding and upstream portion of *LCB2*, into the corresponding sites of the polylinker in pRS305 (*LEU2*) (15).

YEpLCB1-3 was constructed by first blunt-end ligating a 365-bp *Sau*3AI–*Dde* I DNA fragment carrying the *GAL1-10 UAS* (16) into the *Hpa* I site located in the *LCB1* promoter. This modified *LCB1* gene was inserted, as a 3.4-kb *Cla* I–*Nru* I DNA fragment, into YEp429 (17) cut with *Cla* I and *Nru* I. YEpLCB2-2 was constructed by inserting the 365-bp *GAL1-10 UAS* fragment into the *Sma* I site of the *LCB2* promoter and then transferring the modified gene as an *Eco*RI fragment into the *Eco*RI site of YEp430 (17).

Several multicopy vectors were used to overproduce the Lcb1 and Lcb2 proteins in yeast. pLCB1-4 was constructed by inserting *LCB1*, as a 2.8-kb *Stu* I–*Bam*HI chromosomal fragment, into YEp429 cut with *Nru* I and *Bam*HI. pLCB2-4 was made by inserting *LCB2* as a 4.3-kb *Eco*RI chromosomal fragment into YEp429 cut with *Eco*RI. pLCB2-6 was made by inserting *LCB1*, as a 2.8-kb *Bam*HI–*Bgl* II fragment, into pLCB2-4 cut with *Bam*HI.

Miscellaneous Procedures. *S. cerevisiae* cells were transformed by using a lithium acetate procedure (18). The following procedures have been described: genetic crosses and tetrad analysis (18), assay of SPT activity (13), and growth inhibition of yeast cells by sphingofungin B (19). For inhibition assays, the microtiter wells contained 200 μl of medium and growth was scored after 3–6 days of incubation at 30°C. Both strands of DNA were sequenced from double-stranded DNA templates using Sequenase version 2.0 DNA (United States Biochemical) and synthetic oligonucleotide primers as described in the Sequenase kit.

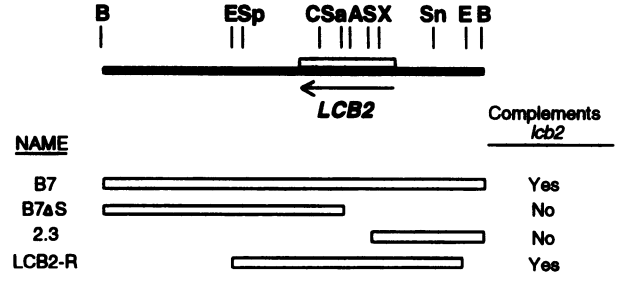

FIG. 1. Diagram of the *LCB2* locus. A restriction map of the *LCB2* chromosomal region is shown at the top of the figure with the *LCB2* open reading frame indicated above the solid bar and the direction of transcription by the arrow. DNA fragments used to localize the *LCB2* gene are indicated below as is their ability to complement the Lcb⁻ phenotype of an *lcb2* mutant strain.

RESULTS

Isolation of LCB2. The *LCB2* gene was isolated from a *S. cerevisiae* genomic DNA library (12) by complementation of the Lcb⁻ phenotype of strain BS238 (*lcb2*). Ura⁺ transformants were selected, pooled, and reselected on plates lacking phytosphingosine to obtain Lcb⁺ transformants. Plasmid DNA, recovered from several Lcb⁺ cells by transformation into *Escherichia coli*, gave an identical restriction fragment pattern, indicating that all plasmids carried the same 7-kb insert, designated B7 (Fig. 1).

The *LCB2* gene was localized by subcloning and testing the subclones for complementation of the Lcb⁻ phenotype in strain BS238. This approach localized the gene to a region near the *Apa* I site as shown in Fig. 1. The DNA sequence around this site was determined and the sequence was scanned by computer for open reading frames. Only one large open reading frame was found as indicated in Fig. 1.

To prove that this open reading frame was necessary for long-chain base synthesis, we first replaced one of the two *LCB2* alleles in the diploid strain YPH501 with the *lcb2-Δ3* deletion allele. Diploids were sporulated and spores were tested for their Lcb phenotype. All 17 four-spored tetrads segregated 2Lcb⁺:2Lcb⁻ spores and all the Lcb⁻ spores were Ura⁺, as expected because the deletion allele was made using the *URA3* gene. These results demonstrate that the open reading frame is necessary for synthesis of the long-chain base component of sphingolipids as would be expected if the open reading frame was the *LCB2* gene.

To verify that the open reading frame was allelic to *LCB2*, we used the integrating vector pRSLCB2-2, which only carries the 5′ half of the putative *LCB2* gene. The plasmid was directed, by digestion within the coding region using the restriction endonuclease *Nsi* I, to integrate by homologous recombination into the *lcb2* locus of strain BS238. Integration at the correct chromosomal location was verified by Southern blot analysis (data not shown). The resulting strain (relevant phenotype Lcb⁻) carrying the integrated plasmid was crossed to strain YPH500 (relevant phenotype Lcb⁺), and diploids were selected by auxotrophic complementation and sporulated. Twenty-five four-spored tetrads segregated 2Lcb⁺:2Lcb⁻ spores, all of the Lcb⁺ spores were Leu⁻, and the Lcb⁻ spores were Leu⁺ as expected if pRSLCB2-2 (relevant phenotype Leu⁺) had integrated at or near the *lcb2* allele. These data plus the preceding data demonstrate that the *LCB2* gene has been cloned.

Analysis of the Predicted Lcb2 Protein. The Lcb2 protein (LCB2, Fig. 2) is predicted to contain 561 amino acid residues (M_r = 63,110) and to have an isoelectric point of 8.06. Analysis of the hydropathy profile (25) predicts two transmembrane segments (residues 58–78 and 443–461). Two other algorithms (26, 27) predict that residues 57/58 to 77/78 have a high probability of forming a membrane-associated helix, whereas residues 443–463 have a lower probability.

The databases at the National Center for Biotechnology Information were scanned for amino acid sequences similar to the Lcb2 sequence by using the BLAST algorithm (28). Several proteins showed similarity (Fig. 2) as did the 5-aminolevulinic acid synthases from many species (data not shown) besides *S. cerevisiae*.

We also found the 3′ end of an open reading frame upstream of and in the same orientation as *LCB2* that is predicted to encode a protein homologous to the ABC family of membrane transporters (29).

Chromosomal Location of *LCB2*. *LCB2* was mapped distal to *trp1* on chromosome IV by hybridization of the *LCB2* coding region to a set of phage λ clones whose *S. cerevisiae*

FIG. 2. Protein sequence comparison. The *S. cerevisiae* LCB2 protein sequence was aligned with other pyridoxal phosphate-dependent amino acid acyltransferases by using the CLUSTAL algorithm (20). Residues identical to LCB2 are shaded black and conserved residues are shaded gray. Gaps in a sequence are indicated by dash. The sequences are *E. coli* 8-amino-7-oxononanoate synthase [BIOF (21)], *E. coli* 2-amino-3-ketobutyrate CoA ligase [AKBL (22)], *S. cerevisiae* 5-aminolevulinic acid synthase [HEM1 (23)], and the *S. cerevisiae* SPT subunits [LCB2 and LCB1 (12)]. Similar amino acids are A, S, and T; D and E; N and Q; R and K; I, L, V, and M; F, Y, and W. Underlined residues 58–78 indicate a potential transmembrane segment in LCB2. The star above the sequences indicates a lysine residue in the AKBL protein that forms a Schiff base with pyridoxal phosphate (24).

DNA inserts have been mapped to the yeast genome (30). The probe hybridized to λ clone 6248 (American Type Culture Collection). This assignment was verified by a genetic cross between strains GRF123 and BS238. Tetrad analysis showed that *trp1* and *rrp1* were separated by 120 centimorgans, *trp1* and *lcb2* were separated by 63 centimorgans, and *rrp1* and *lcb2* were separated by 7.7 centimorgans, indicating the order *trp1–lcb2–rrp1*; analysis of the double recombinants gave the same order. Previous genetic data indicated that *rrp1* mapped very close to *mak21*, between *trp1* and *rad55* (14), but this order was inconsistent with the physical map of the chromosome and with our genetic data that indicated the order was *CEN–trp1–lcb2–rad55–rrp1*. This conundrum was resolved by showing that *RRP1* hybridized to λ clone 4012, which placed *rrp1* distal to *rad55* on the physical map of chromosome IV rather than between *trp1* and *rad55*.

The *LCB1* hybridized to λ clone 2988, which overlaps clone 4467. Thus, *LCB1* maps to chromosome XIII between *SUP8* and *ade4*.

The *LCB2* Gene Restores SPT Activity. Strains defective in *lcb2* lack SPT activity (13). Data presented in Table 2 demonstrate that the *LCB2* gene, but not the *LCB1* gene, restored SPT activity in an *lcb2* mutant strain. The gene did not restore SPT activity in an *lcb1* mutant strain (data not shown).

Overexpression of *LCB1* and *LCB2*. Lack of SPT activity in a strain defective in *lcb1* or *lcb2* combined with the protein similarity data suggests that the two genes encode subunits of SPT. A prediction of this hypothesis is that overexpression of both genes should increase SPT activity. This prediction was verified. Wild-type *S. cerevisiae* transformed with a multicopy vector carrying both *LCB1* and *LCB2* gave 2.5-fold more SPT activity than did the strain transformed with a vector alone or with a plasmid carrying only one *LCB* gene (Table 2): Data (not shown) from other experiments showed a 3- to 4-fold difference.

An indirect assay was used to measure the amount of the Lcb1 and Lcb2 proteins because no antibody to the proteins is available. The assay used sphingofungin B, a compound that resembles SPT substrates and prevents growth of *S. cerevisiae* by inhibiting SPT activity (19). The data presented in Table 3 show that greatest resistance to sphingofungin B

Table 2. SPT activity in *S. cerevisiae* strains

Strain (relevant genotype)	Transforming plasmid (*LCB* gene)	SPT activity
YPH250 (*LCB2*)	None	173
LCB25 (*lcb2-Δ4*)	YEpLCB1-3 (*LCB1*)	0
LCB25 (*lcb2-Δ4*)	YEpLCB2-2 (*LCB2*)	162
LCB25 (*lcb2-Δ4*)	YEp430 (vector)	7
YPH102	pLCB2-6A (*LCB1 LCB2*)	358
YPH102	pLCB2-4 (*LCB2*)	84
YPH102	pLCB1-4 (*LCB1*)	130
YPH102	YEp429 (vector)	144

Activity is expressed as pmol per min per mg of protein. Cells were cultured on defined medium containing 12 μM phytosphingosine and lacking uracil, histidine, or leucine as necessary to select for the transforming plasmid. Total membranes were prepared as described (13) except that cell breakage with glass beads was carried out using a Braun homogenizer. SPT activity was measured as the palmitoyl-CoA-dependent conversion of labeled serine to chloroform-soluble radioactivity by method I (13) except that the palmitoyl-CoA concentration was increased to 160 μM. For YPH102 transformants, the product of the reaction was verified and quantified by HPLC (13).

Table 3. Resistance to growth inhibition by sphingofungin B

Plasmid	*LCB* gene	MIC
pLCB2-6	*LCB1* and *LCB2*	8×, 16×, 32×
pLCB1-4	*LCB1*	2×, 2×
pLCB2-4	*LCB2*	1×, 2×
YEp429	None	1×, 1×, 1×

Strain YPH102, transformed with the indicated plasmid, was assayed to determine the minimum inhibitory concentration (MIC) of sphingofungin B. Values from two or three experiments are expressed relative to the strain transformed with YEp429, which was inhibited by 3.12 μg of sphingofungin B per ml (1×).

was obtained in a strain carrying both *LCB* genes on a multicopy vector; less resistance was obtained with a multicopy vector carrying one *LCB* gene. These data argue that each Lcb protein is overproduced 8- to 32-fold in the strain carrying both *LCB* genes and that maximum sphingofungin resistance requires overproduction of both proteins.

DISCUSSION

Mutation of the *LCB1* or the *LCB2* gene had been shown to produce the same phenotype (13). Since the *LCB1* gene appeared to encode SPT or a subunit of the enzyme (12), it seemed likely that *LCB2* might do the same. The data presented in this paper support the hypothesis that the Lcb2 protein is also a subunit of SPT. (*i*) The Lcb2 protein is predicted to be most closely related to Lcb1 protein (21.6% amino acid identity, 43% similarity) and only slightly less related to the enzymes that are related to Lcb1 protein (Fig. 2 and ref. 12), all of which are pyridoxal phosphate-utilizing homodimeric enzymes that catalyze the transfer of the acyl group from an acyl-CoA to the α-carbon of an amino acid. (*ii*) Increased SPT activity was only obtained when both *LCB* genes were present in multiple copies in yeast cells (Table 2). (*iii*) Resistance to the SPT inhibitor sphingofungin B (19) was greatest when both *LCB* genes were present in multiple copies (Table 3). Sphingofungin B most likely acts by binding to the catalytic site of SPT, implying then that both Lcb proteins are necessary for formation of the catalytic site. (*iv*) Yeast SPT activity is membrane-bound (31) and both Lcb proteins are predicted to have transmembrane segments. These data are not consistent with an alternative hypothesis that one or the other Lcb protein regulates the concentration of SPT. However, further data, particularly subunit analysis of SPT, as yet to be purified, will be needed to prove that both Lcb proteins are SPT subunits.

Analysis of the derived Lcb2 protein identified a sequence, G^{362}TFTKSFG^{369} (Fig. 2), that is related to a pyridoxal phosphate binding motif found in the *E. coli* 2-amino-3-ketobutyrate CoA ligase enzyme, where the underlined lysine residue has been shown to form a Schiff base with pyridoxal phosphate (24). This motif seems to be part of a larger motif, (D/E)XXXX(S/T)XXKX(L/F)GXXGG(F/Y), found in the enzymes shown in Fig. 2 and in other aminolevulinic acid synthetases. The derived Lcb1 protein sequence has a threonine in place of the underlined lysine, which suggests that Lcb2 protein binds the coenzyme. Lcb1 protein may also participate directly in catalysis or it may be a regulatory subunit.

A major unsolved problem in the field of sphingolipid research is how the concentration of sphingolipids is regulated. Our results indicate that one form of regulation may modulate SPT activity. SPT activity increased 2- to 4-fold when both *LCB* genes were carried on a multicopy vector (Table 2) but the data from the sphingofungin B experiments (Table 3) indicated that the Lcb1 and Lcb2 proteins were overproduced 8- to 32-fold and Northern blot analysis indicated a 3- to 6-fold increase in mRNA (data not shown). Thus, *S. cerevisiae* may have a way to limit SPT activity even when the SPT subunits are present in excess.

Recent results from Zhao *et al.* (32) portend a complex regulatory process and demonstrate that suppressors of the Ca^{2+}-sensitive phenotype of the yeast mutant *csg2* fall into seven complementation groups, one of which is *LCB2*. Sphingolipid synthesis is abnormal in all seven complementation groups. These results imply an unsuspected relationship between sphingolipids and Ca^{2+} metabolism that involves the Lcb2 protein.

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