1-1-2000

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 11, Issue of March 17, pp. 7597–7603, 2000
Printed in U.S.A.

Tsc3p Is an 80-Amino Acid Protein Associated with Serine Palmitoyltransferase and Required for Optimal Enzyme Activity*

(Received for publication, November 16, 1999, and in revised form, December 8, 1999)

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Serine palmitoyltransferase catalyzes the first step of sphingolipid synthesis, condensation of serine and palmitoyl CoA to form the long chain base 3-ketosphinganine. The *LCB1/TSC2* and *LCB2/TSC1* genes encode homologous proteins of the α-oxoamine synthase family required for serine palmitoyltransferase activity. The other α-oxoamine synthases are soluble homodimers, but serine palmitoyltransferase is a membrane-associated enzyme composed of at least two subunits, Lcb1p and Lcb2p. Here, we report the characterization of a third gene, *TSC3*, required for optimal 3-ketosphinganine synthesis in *Saccharomyces cerevisiae. S. cerevisiae* cells lacking the *TSC3* gene have a temperature-sensitive lethal phenotype that is reversed by supplying 3-ketosphinganine, dihydrosphingosine, or phytosphingosine in the growth medium. The *tsc3* mutant cells have severely reduced serine palmitoyltransferase activity. The *TSC3* gene encodes a novel 80-amino acid protein with a predominantly hydrophilic amino-terminal half and a hydrophobic carboxyl terminus that is membrane-associated. Tsc3p coimmunoprecipitates with Lcb1p and/or Lcb2p but does not bind as tightly as Lcb1p and Lcb2p bind to each other. Lcb1p and Lcb2p remain tightly associated with each other and localize to the membrane in cells lacking Tsc3p. However, Lcb2p is unstable in cells lacking Lcb1p and *vice versa*.

Serine palmitoyltransferase (EC 2.3.1.50) (SPT)[1] catalyzes the formation of 3-ketosphinganine from serine and palmitoyl CoA. This is the first committed step in the synthesis of ceramides and sphingolipids. This step is also believed to be rate-limiting, making it likely that regulation of SPT controls the rate of sphingolipid synthesis. For example, treatment of mammalian cells with sphingosine results in down-regulation of SPT activity (1). However, little is known about how the enzyme is regulated.

Two genes, *LCB1/TSC2* and *LCB2/TSC1*, are required for SPT activity (2–4). Both genes encode proteins that belong to a small subfamily of pyridoxal 5′-phosphate-dependent enzymes that catalyze the condensation of an amino acid and a carboxylic acid CoA thioester with concomitant decarboxylation of the amino acid. This α-oxoamine synthase subfamily includes 8-amino-7-oxononanoate synthase (AONS), 5-aminolevulinate synthase, 2-amino-oxobutyrate CoA ligase, and SPT. Although these enzymes share low overall sequence identity, the recently reported crystal structure of AONS reveals that several functionally important residues are highly conserved (5). The residues that are involved in pyridoxal phosphate binding, including the lysine that forms a Schiff's base with pyridoxal phosphate, are conserved in Lcb2p but not in Lcb1p. Therefore, although the AONS enzyme is a symmetrical homodimer with the active site at the subunit interface, Lcb1p and Lcb2p may form a heterodimer because both proteins are required for SPT activity. The yeast and mammalian Lcb2ps are more similar to each other than they are to the yeast and human Lcb1ps (2, 6, 7). Likewise, the yeast and mammalian Lcb1ps are more similar to each other than they are to their Lcb2p counterparts (7, 8).

SPT also differs from the other members of this enzyme family because it is membrane-associated. SPT appears to be located on the cytoplasmic side of the endoplasmic reticulum (9). In comparison to AONS, Lcb1p and Lcb2p each have amino-terminal extensions that contain potential membrane-spanning segments. However, it is not known whether these hydrophobic segments are important for membrane association.

Attempts to increase SPT activity by over-expression of *LCB1* and *LCB2* have met with limited success (3), raising the possibility that additional proteins may be required for SPT activity. Many genes involved in sphingolipid synthesis have been identified through the characterization of suppressors of the Ca^{2+} sensitivity of *csg2*Δ mutants (2, 10–14). Csg2p is required for mannosylation of the sphingolipid intermediate inositolphosphorylceramide; therefore, cells lacking Csg2p overaccumulate inositolphosphorylceramide, which in turn confers Ca^{2+} sensitivity (15). The *tsc* (for temperature-sensitive suppressors of Ca^{2+} sensitivity) mutants were isolated by first selecting pseudorevertants of the *csg2*Δ mutant that could grow on medium containing 50 mM $CaCl_2$ at 26 °C. In a secondary screen, the subset of mutants with suppressing mutations that conferred temperature-sensitive lethality (inability to grow at 37 °C) was identified (10). Here, we report the characterization of the *TSC3* gene and show that it is required for optimal SPT activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth—The yeast strains used in this study are listed in Table I. The *lcb1*Δ, *lcb2*Δ, and *tsc3*Δ null mutants (with their respective genes deleted) were constructed in TDY 2039. Media were prepared and cells were grown according to standard procedures (16). The *lcb1*Δ and *lcb2*Δ mutant cells were grown in medium containing phytosphingosine (PHS) at 10–25 μM, which was added to autoclaved

* This work was supported by National Institutes of Health Grant GM 51891 and Uniformed Services University of the Health Sciences Grant CO71DC. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SPT, serine palmitoyltransferase; LCB, long chain base; AONS, 8-amino-7-oxononanoate synthase; ts, temperature sensitive; bp, base pair(s); PHS, phytosphingosine; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; HA, hemagglutinin; PCR, polymerase chain reaction; SCS, suppressor of the Ca^{2+}-sensitive phenotype of the *csg2*Δ mutant.

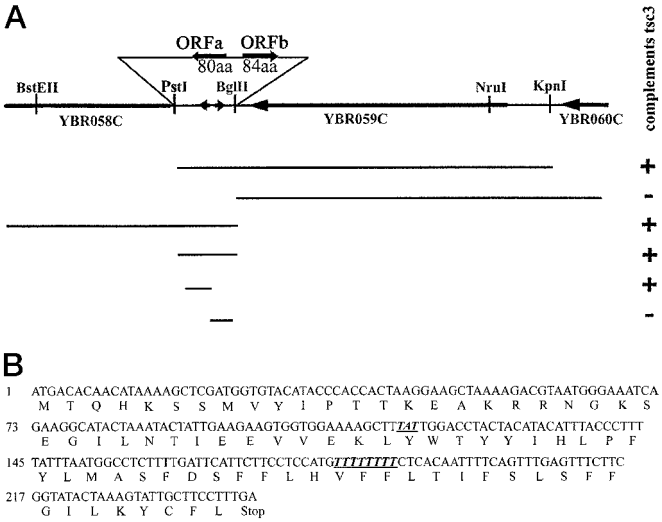

FIG. 1. **The *TSC3* gene lies between YBR058c and YBR059c.** *A,* the complementing genomic library plasmids contained the indicated region of chromosome 2. Subclones were constructed and tested for complementation of the ts phenotype of a *tsc3* mutant. A + symbol denotes that the subclone complements, and a – symbol denotes that the subclone fails to complement. *B,* the 240-base pair open reading frame and the encoded 80-amino acid Tsc3p protein are shown. The stretch of eight thymidine residues where four of the *tsc3* mutations reside and the Tyr codon 39 that is mutated to a stop codon in the *tsc3–2* allele are italicized and *underlined*.

medium containing 0.2% tergitol from a 25 mM PHS stock prepared in ethanol.

Cloning TSC3—The CJYa16 (*tsc3-2*) mutant cells were transformed with a YCp50-based genomic library (17), and transformants that regained the ability to grow at 37 °C were selected. Plasmids were recovered from six temperature-resistant transformants and the genomic fragments on the plasmids were sequenced. The complementing sequences all contained an overlapping region of chromosome 2 including the amino terminus of YBR058c, YBR059c, the carboxyl terminus of YBR060c, and two additional small ORFs between 059c and 058c with the potential to encode an 80-amino acid protein and an 84-amino acid protein (Fig. 1*A*).

Subcloning to Identify the tsc3-Complementing ORF and Sequencing the tsc3 Mutant Alleles—Subclones were constructed by inserting the indicated restriction fragments into pRS316 (18) (Fig. 1*A*). The two potential small ORFs from this region (labeled ORFa and ORFb in Fig. 1*A*) were amplified by PCR and cloned into pRS316. For the 80-amino acid ORFa, a 380-bp *Xho*I/*Bam*HI-ended fragment was generated using primers 5′-GGCCCTCGAGGCTCGCAATTTGACAGAA and 5′-GGCCGGATCCTTGCCTCCAGCTTATACTA. For the 84-amino acid ORFb, a 460-bp *Pst*I/*Bam*HI-ended fragment was generated using primers 5′-GGCCCTGCAGTAGTATTTAGTATGCCTTC and 5′-GGCCGGATCCGTAGTGCATCCAGTAGTGG. The subclones were tested for the ability to complement the *tsc3-2* mutant (Fig. 1*A*). Genomic DNA was isolated from each of the five mutants in the TSC3 complementation group and used as the template for PCR amplification of the mutant alleles using the primers for ORFa. The fragments were sequenced.

Disruption of TSC3, LCB1, and LCB2—The coding sequence of *TSC3* was deleted and substituted with a *TRP1* marker gene. This was accomplished by generating two restriction fragments that contained the flanking sequences of *TSC3* by PCR. The fragment containing the upstream flanking sequence was *Kpn*I/*Xho*I-ended and extended from about 200 bp before the start codon to 30 bp past the start codon, and the downstream flanking fragment was *Xho*I/*Eco*RI-ended and extended from about 40 bp before the stop codon to 300 bp past the stop codon. These fragments were ligated together and inserted between the *Kpn*I and *Eco*RI sites of pUC19, yielding a plasmid having a *Xho*I site replacing most of the TSC3 coding sequence. A PCR-generated *Sal*I-ended TRP1 fragment was ligated into the *Xho*I site, and the disrupting allele was liberated by digestion with *Kpn*I and *Eco*RI. The disrupting allele of the *LCB1* gene was constructed by replacing the 708-bp *Msc*I fragment (internal to the *LCB1* gene) with a PCR-generated *TRP1* marker gene in a pUC19-based plasmid containing a 2050-bp *Cla*I to *Hin*dIII fragment that includes the entire *LCB1* gene. Construction of the disrupting allele of *LCB2* has been described (2).

Preparation of Microsomes—Cells in early exponential growth were pelleted, washed with H_2O, and resuspended at 2 ml/g wet cell weight in a buffer composed of 50 mM Tris, 7.5, 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A. Glass beads were added to just below the meniscus, and cells were lysed by six cycles (60 s each) of vortexing with cooling on ice between cycles. Unbroken cells, beads, and debris were removed by centrifugation (5000 × *g* for 10 min), and the low speed supernatant was centrifuged at $4 \times 10^4 \times g$ for 40 min to provide the microsomal pellet. The pellet was resuspended at 1 ml/g wet cell weight (~10 mg/ml protein) in the same buffer containing 33% glycerol and stored at −80 °C.

Assay of Serine Palmitoyltransferase Activity—SPT activity was assayed in a 300-μl volume containing 50 mM HEPES, 8.3, 50 mM pyridoxal phosphate, 2 mM serine (20 μCi/ml) 0.2 mM palmitoyl CoA (or as indicated in the legend to Fig. 3), 1 mM NADPH, 2.4 mM glucose-6-phosphate, and 10 units of glucose-6-phosphate dehydrogenase. The reaction was initiated by adding 0.5–1 mg of microsomal protein, and after 10 min at 37 °C, it was terminated by the addition of 100 μl of 2 M NH_4OH and 0.75 ml of $CHCl_3$:MeOH (1:2). After vortexing, an additional 0.75 ml of $CHCl_3$:MeOH (1:2), 1 ml of $CHCl_3$, and 2 ml of 0.5 M NH_4OH were added with vortexing after each addition. After brief centrifugation to separate the phases, the top layer was aspirated off. The organic phase was washed three times (or until clear) with 30 mM KCl, and a fixed volume was dried under N_2 and resuspended in scintillation fluid for counting. SPT activity is expressed in pmol of serine converted per mg of microsomal protein per min.

Generation of the Anti-Lcb1p and Anti-Lcb2p Polyclonal Antibodies—Lcb1p and Lcb2p were expressed in *Escherichia coli* using the pET-19b vector (Novagen). For this purpose, the entire coding sequences of the genes were PCR-amplified using 5′-GCCATGG**CATATG**GCACACATCCCAGAGGT and 5′-GCTAGC**GGATCC**TTTATTAGATTCTTGGCAAC as primers for LCB1, and 5′-GCCATGG**CATATG**AGTACTCCTGCAAACTA, and 5′-GCTAGC**GGATCC**ATTAACAAAATACTTGTCGT as primers for *LCB2*. The PCR products were digested with *Nde*I and *Bam*HI (boldface and underlined), ligated into the pET-19b vector, and the resulting plasmids were transformed into *E. coli* BL21 cells. Induction of the T7 promoter yielded high level expression of the two proteins, and they fractionated with the inclusion bodies. The proteins were purified by preparative SDS gel electrophoresis for use as immunogens. Antibodies to the recombinant Lcb1p and Lcb2p were generated by Cocalico Biologicals Inc. (Reamstown, PA).

Epitope Tagging the TSC3 and LCB1 Genes—Two amino acid insertions were introduced across the coding sequence of the *TSC3* gene by placing 6-bp *Nhe*I sites after codon 12, 28, 54, 69, or 79 using the QuikChange site-directed mutagenesis kit (Stratagene). The mutated alleles were tested for complementation of the ts lethality of the *tsc3*Δ mutant, and it was found that two amino acids insertions after either codon 12 or codon 28 (but not at the other sites) resulted in functional *TSC3* alleles. A *Spe*I-ended triple-hemagglutinin (HA)-containing cassette was generated by PCR and ligated in-frame into the *Nhe*I site between codons 12 and 13. A plasmid containing the *TSC3-HA* allele (confirmed by DNA sequencing) was found to be functional on the basis of complementation of the ts phenotype of the *tsc3*Δ mutant. An Lcb1p-GST fusion protein was constructed using a similar strategy. First, an *Avr*II site was introduced into the *LCB1* gene by changing codons 9 and 10 from CCCAAA to CCTAGG by QuikChange mutagenesis, and then a *Spe*I-ended GST-containing fragment was generated by PCR and ligated into the *Avr*II site. The construct was confirmed by DNA sequencing, and the *LCB1* allele containing GST between codons 9 and 10 was demonstrated to be functional on the basis of complementation of the lethality of the *lcb1*Δ mutant and restoration of SPT activity to membranes prepared from the rescued null mutant.

Immunoblotting—Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and the blots were blocked in 0.1 M Tris, 7.5, 0.15 M NaCl, 0.1% Tween 20, 5% dry milk. For detecting Lcb1p or Lcb2p, blots were probed with anti-Lcb1p or anti-Lcb2p rabbit polyclonal antibodies (1/500) followed by a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1/2000) (Amersham Pharmacia Biotech). Tsc3p-HA was detected using monoclonal anti-HA (12CA5, Berkeley Antibody Co., Berkeley, CA) at 1/200 as the primary antibody, and goat horseradish peroxidase-conjugated anti-mouse (Amersham Pharmacia Biotech) at 1/2000 as the secondary antibody. The bound antibodies were detected by with the ECL Western blotting detection system (Amersham Pharmacia Biotech).

Immunoprecipitation—The microsomes were solubilized at 1 mg/ml with 2 mM sucrose monolaurate (Roche Diagnostics, Indianapolis, IN) for 10 min, and the high speed ($1 \times 10^5 \times g$, 30 min) supernatant was collected. The solubilized microsomes (150 μl) were incubated with 3 μl

of the precipitating antibody for 2 h, and then 20 μl of protein A-Sepharose (125 mg/ml from Sigma) for 2 h. The precipitates were washed three times with 600 μl of 50 mM HEPES, pH 7.5, and resuspended in 150 μl of SDS loading buffer, and a 10-μl sample was subjected to SDS-PAGE and immunoblot analysis. In some cases, NaCl was included during the immunoprecipitation.

RESULTS

Isolation of the tsc3 Mutants and Cloning the TSC3 Gene—A collection of suppressors of the Ca^{2+}-sensitive phenotype of the *csg2*Δ mutant is being characterized. Each of these suppressor mutants (the TSC collection) has a single mutation that suppresses Ca^{2+} sensitivity and confers temperature-sensitive (ts) lethality (10). The five mutants in the TSC3 complementation group have phenotypes indicative of defects in SPT activity, and therefore, we undertook a study of the *TSC3* gene. The suppressing and ts phenotypes of one of the mutants in the TSC3 complementation group are shown in Fig. 2*B*.

The five *tsc3csg2*Δ mutants (Table I) were mated with the original *csg2*Δ single mutant. The resulting *tsc3*/*TSC3 csg2*Δ/*csg2*Δ diploids were both ts and Ca^{2+}-sensitive, indicating that both the suppressing and the ts phenotypes of the *tsc3* mutant were recessive. Following sporulation and tetrad analysis, half of the products of meiosis were found to be ts and resistant to Ca^{2+}, and the other half were temperature-tolerant and Ca^{2+}-sensitive, demonstrating that a single mutation caused both the suppressing and the ts phenotypes. During tetrad analysis, genetic linkage between the *TSC3* locus and the *LYS2* gene was fortuitously observed. Therefore, the linkage distance between *TSC3* and three other genes in that region of chromosome 2 (*CSG2*, *LYS2,* and *SEC18*) was measured. The *TSC3* locus mapped to a 30-kilobase region on chromosome 2 in the vicinity of YBR058c (data not shown).

The *TSC3* gene was cloned by complementation of the ts phenotype of the *tsc3-2* mutant (see under "Experimental Procedures"). The complementing genomic fragment contained parts of YBR058c and YBR060c and all of YBR059c (Fig. 1*A*). The *TSC3* gene was expected to lie in this region of chromosome 2 based on the genetic linkage of the *TSC3* locus with markers from this region. Subcloning experiments demonstrated that the intergenic region between YBR058c and YBR059c was responsible for complementing the *tsc3* mutants (Fig. 1*A*). This region has two potential small ORFs unannotated in the *Saccharomyces* Genome Data Base due to their small (<100-amino acid) sizes. Fragments containing each of the ORFs were amplified by PCR and inserted into pRS316 (18). The plasmid containing only ORFa, (Fig. 1*A*), which potentially encodes an 80-amino acid protein, complemented the ts phenotype of the *tsc3* mutants.

The five *tsc3* mutants each have nucleotide changes that alter the coding potential of the 240-bp ORFa providing further evidence that ORFa encodes Tsc3p. Four of the five *tsc3* alleles have mutations in the same stretch of 8 thymidine residues (codons 61, 62, and 63) in the 240-bp ORF (Fig. 1*B*). The *tsc3-1* and *tsc3-4* mutations have an extra T resulting in a new reading frame for 39 amino acids prior to encountering a termination codon. The *tsc3-3* mutation has a deletion of one of the T residues, leading to a frameshift mutation that alters 4 amino acid residues before causing premature termination. The *tsc3-5* allele has an extra CT at the end of the stretch of 8 thymidines, also causing a frameshift mutation that alters 5 amino acids before a premature termination. The point mutation in the *tsc3-2* allele changes the tyrosine codon 39 to a stop codon (Fig. 1*B*).

The possibility that the 80-amino acid protein potentially encoded by the *TSC3* gene might not be expressed was considered. For example, *TSC3* could express a regulatory RNA. The single nucleotide change that converts codon 39 to a stop codon and disrupts function of the *TSC3* gene suggested that the ORF is translated. To provide further evidence that the encoded 80-amino acid protein is responsible for complementation of the

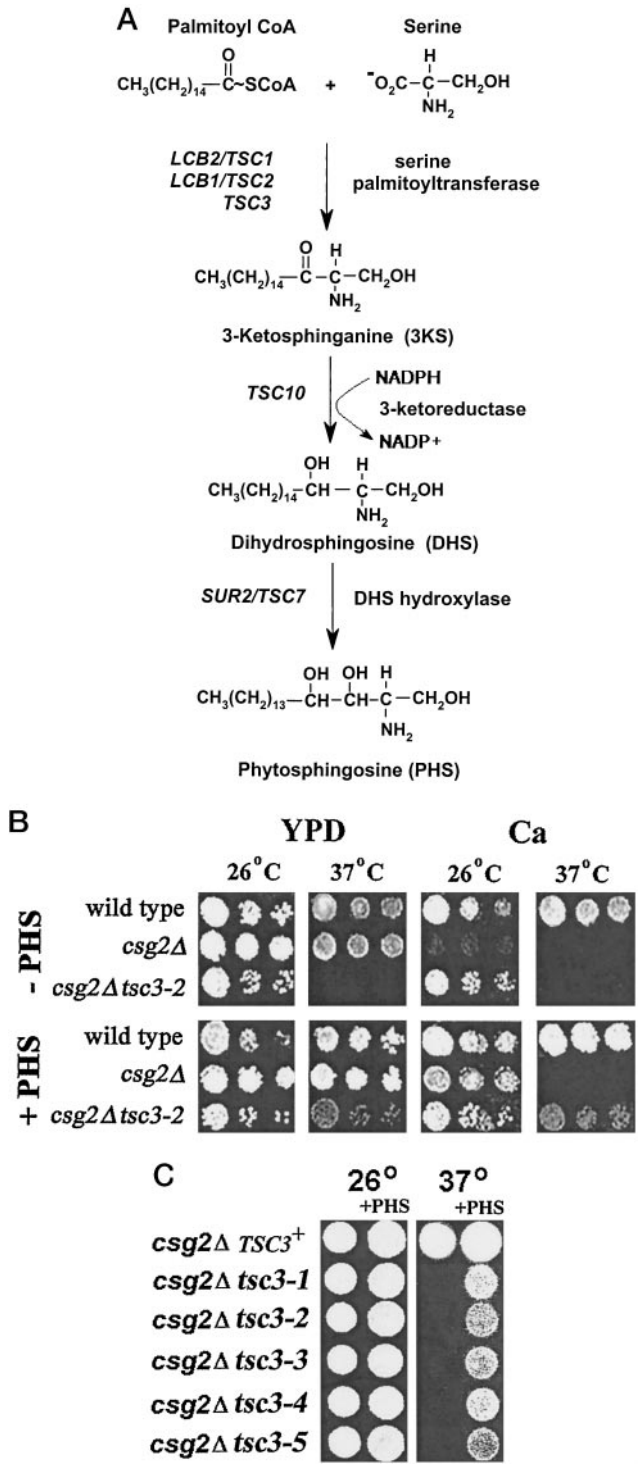

FIG. 2. **Mutations in the *TSC3* gene suppress the Ca^{2+} sensitivity of *csg2*Δ mutants and confer temperature-sensitive lethality.** The temperature sensitivity is reversed by phytosphingosine. *A,* the pathway for synthesis of the long chain bases is shown. *B,* the growth phenotypes of wild-type (TDY 2039), *csg2*Δ (TDY 2040), and *tsc3–2 csg2*Δ (CJYa16) mutant cells are compared. Serial dilutions of the cells were transferred from the wells of a microtiter plate to YPD agar plates (*YPD*) with or without 50 mM $CaCl_2$ (*Ca*), and with or without 20 μM PHS. The plates were incubated at either 26 °C (3 days) or 37 °C (2 days). *C,* the ts growth phenotype of all five *tsc3* mutants is rescued by PHS. Growth on PHS is shown, but 3-ketosphinganine and dihydrosphinganine also reverse the ts phenotype.

TABLE I
S. cerevisiae strains

Strain	Genotype
TDY 2037	*Matα lys2 ura3–52 trp1Δ leu2Δ*
TDY 2038	*Matα lys2 ura3–52 trp1Δ leu2Δ csg2::LEU2*
TDY 2039	*Mata ade2–101 ura3–52 trp1Δ leu2Δ*
TDY 2040	*Mata ade2–101 ura3–52 trp1Δ leu2Δ csg2::LEU2*
LHYa60	*Mata tsc3–1 ade2–101 ura3–52 trp1Δ leu2Δ csg2::LEU2*
CJYa16	*Mata tsc3–2 ade2–101 ura3–52 trp1Δ leu2 Δ csg2::LEU2*
CJYa18	*Mata tsc3–3 ade2–101 ura3–52 trp1Δ leu2Δ csg2::LEU2*
CJYα8	*Matα tsc3–4 lys2 ura3–52 trp1Δ leu2Δ csg2::LEU2*
CJYα11	*Matα tsc3–5 lys2 ura3–52 trp1Δ leu2Δ csg2::LEU2*

tsc3 mutants, single nucleotide changes were introduced at codon 7, 24, 50, or 69, converting each to a stop codon. None of these mutated *TSC3* alleles complemented the ts phenotype of the *tsc3* mutants; therefore, it is concluded that expression of the 80-amino acid protein is necessary for reversing the ts lethality of the *tsc3* mutants. The 80-amino acid protein encoded by *TSC3* has a hydrophilic amino-terminal half and a hydrophobic carboxyl-terminal half (Fig. 1*B*). No proteins with significant homologies to Tsc3p were identified in a BLAST search against the complete protein sequence data bases (nonredundant data base, GenBank™).

The TSC3 Gene Is Essential for Growth at High Temperatures—The nature of the *tsc3* mutant alleles suggested that the ts and suppressing phenotypes result from eliminating, rather than altering, the function of Tsc3p. For example, the *tsc3-2* mutant has a stop codon at residue 39. To ascertain that expression of the amino-terminal portion of Tsc3p is not required for viability, a null allele having the *TSC3* coding sequence replaced with the *TRP1* gene (see under "Experimental Procedures"), was used to disrupt the *TSC3* gene. The null mutant (*tsc3*Δ) displayed the same phenotype as the originally isolated *tsc3* mutants; it was viable at 26 °C but unable to grow at 37 °C. Therefore, the ts phenotype does not result from the temperature-dependent loss of function of Tsc3p at 37 °C; rather, the ts phenotype arises from the requirement for Tsc3p at high temperature.

The TSC3 Gene Is Required for Optimal SPT Activity—The first three steps in sphingolipid synthesis are bypassed by exogenous phytosphingosine (Fig. 2*A*). Phytosphingosine, dihydrosphingosine, and 3-ketosphinganine all restore growth of the *tsc3* mutants at 37 °C (Fig. 2). This suggested that SPT activity might be deficient in the *tsc3* mutants, and therefore, SPT activity in microsomal membranes isolated from *tsc3* and wild-type cells was compared. The *in vitro* SPT activity measured in the *tsc3* mutants was reduced more than 30-fold in comparison to the SPT activity measured in cells with the wild-type *TSC3* gene (Fig. 3*A*), making it surprising that the *tsc3* mutants are viable. However, further analysis indicates that the percentage of reduction in SPT activity is dependent on the palmitoyl CoA concentrations used for the assay. The conditions that give optimal SPT activity when Tsc3p is present are not the optimal conditions for the Tsc3p-independent enzyme activity. As has been observed previously for the rat enzyme (19), SPT activity increases sigmoidally with increasing palmitoyl CoA concentrations and reaches a maximum at about 0.2–0.3 mM, and at higher palmitoyl CoA concentrations, SPT activity is inhibited (Fig. 3*B*). The Tsc3p-independent SPT activity is inhibited at lower (0.05 mM) palmitoyl CoA concentrations than the SPT activity measured with Tsc3p present

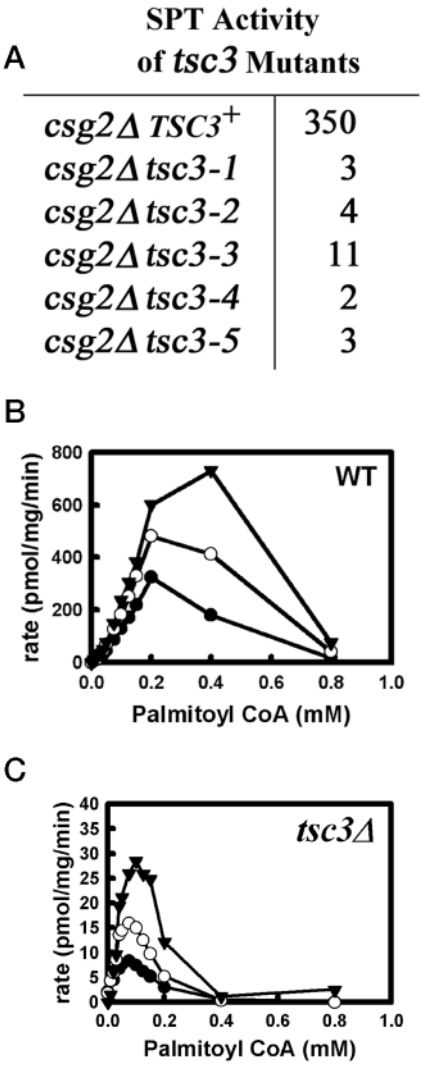

FIG. 3. **The *tsc3* mutants are deficient in SPT activity.** *A,* microsomes were prepared from each of the *tsc3* mutants (Table I), and SPT activity was assayed at 0.2 mM palmitoyl CoA (see under "Experimental Procedures"). SPT activity is reported in pmol of serine converted to 3-ketosphinganine per mg of microsomal protein per min. The SPT activity in wild-type (*B*) and *tsc3*Δ mutant (*C*) microsomes was measured at varying palmitoyl CoA concentrations at serine concentrations of either 2 (●), 6 (○), or 18 (▼) mM.

(Fig. 3*C*). Therefore, the 30-fold difference in SPT activity observed at 0.2 mM palmitoyl CoA is partially related to the difference in substrate inhibition of the enzyme. At low palmitoyl CoA concentrations, the SPT activity in the mutant microsomes is only about 3-fold lower than in the wild-type microsomes.

Tsc3p Is Not Required for Expression, Membrane Association, or Coimmunoprecipitation of Lcb1p and Lcb2p—The possibility that Tsc3p influences SPT activity by affecting the expression of Lcb1p or Lcb2p was investigated. Polyclonal antibodies to Lcb1p and Lcb2p were generated (see under "Experimental Procedures") and used for detecting the Lcb1p and Lcb2p proteins. Although Lcb1p and Lcb2p have very similar predicted molecular weights (62,196 and 63,115, respectively), Lcb1p migrates with a significantly higher apparent molecular weight than Lcb2p (Fig. 4*A*). The calculated pI for Lcb1p is 5.96, whereas that for Lcb2p is 8.43; it is not unusual for proteins with acidic pIs to migrate aberrantly on SDS-PAGE gels. The level of Lcb2p is greatly diminished in lysates prepared from the *lcb1*Δ mutant (Fig. 4*A*). The levels of the *LCB2*

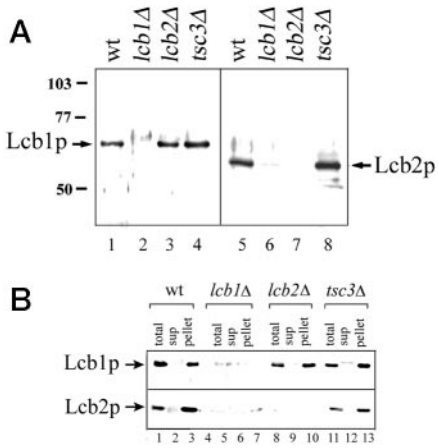

FIG. 4. **Tsc3p is not required for expression, stability, or membrane association of Lcb1p or Lcb2p.** *A,* microsomes were prepared from TDY 2039 (*wt*) or from the *lcb1*Δ, *lcb2*Δ, and *tsc3*Δ mutant cells, which are isogenic to TDY 2039, but with the designated gene disrupted. Ten μg of the microsomal proteins were resolved on 8% SDS-PAGE, and immunoblotted with antibodies to Lcb1p (*left panel*) or antibodies to Lcb2p (*right panel*). Apparent molecular masses of marker proteins and the bands corresponding to the Lcb1p and Lcb2p proteins are indicated. *B,* cell lysates were centrifuged at low speed (4000 × *g* for 10 min) to remove unbroken cells, and the supernatants were then centrifuged at high speed ($4 \times 10^4 \times g$ for 30 min). The proteins from the total cell lysates (*total*) and the supernatants (*sup*) and pellets of the high speed centrifugation were analyzed by immunoblotting with antibodies to either Lcb1p (*top panel*) or Lcb2p (*bottom panel*).

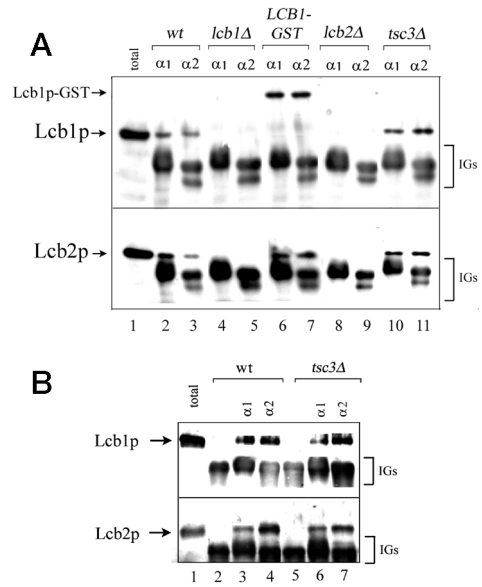

FIG. 5. **Lcb1p and Lcb2p coimmunoprecipitate in wild-type and *tsc3*Δ mutant cells.** *A,* microsomes were prepared from wild-type (*wt*), *lcb1*Δ (with (*lanes 6* and *7*) or without (*lanes 4* and *5*) a plasmid carrying the *LCB1-GST* allele), *lcb2*Δ, and *tsc3*Δ mutant cells as described in the legend to Fig. 4. The microsomes were solubilized with 2 mM sucrose monolaurate, and the $1 \times 10^5 \times g$ (30 min) supernatant was immunoprecipitated using antibodies to either Lcb1p (α1) or Lcb2p (α2). The immunoprecipitated proteins were resolved by 8% SDS-PAGE and analyzed by immunoblotting with the anti-Lcb1p (*top panel*) or anti-Lcb2p (*bottom panel*) antibodies. *Lane 1* contains 10 μg of the microsomes from wild-type cells to mark the position of Lcb1p and Lcb2p. The Lcb1p-GST fusion protein (*lanes 6* and *7*) is larger than Lcb1p. The *dark bands* below Lcb1p and Lcb2p are the rabbit immunoglobulins (*IGs*). *B,* the immunoprecipitations were done using the wild-type and *tsc3*Δ microsomes as described in *A* except that the solubilized microsomes were adjusted to 0.6 M NaCl prior to the immunoprecipitation. Rabbit preimmune serum immunoprecipitation controls are shown in *lanes 2* and *5*.

mRNA are unaltered in the *lcb1*Δ mutant (data not shown), but the rate of translation of Lcb2p in the *lcb1*Δ mutant has not been tested. The genetic and immunoprecipitation data (discussed below) indicate that Lcb1p and Lcb2p are subunits of SPT, and the subunits of a multimeric protein complex are often unstable in the absence of the interacting partners. Furthermore, although Lcb1p is present at normal levels in microsomes prepared from the *lcb2*Δ *m*utant, Lcb1p is unstable in the absence of Lcb2p after the microsomes are solubilized (discussed below). Therefore, it is likely that Lcb2p is synthesized at normal levels but is unstable without Lcb1p.

Tsc3p does not stimulate SPT activity by increasing the levels of the Lcb1p or Lcb2p proteins because the immunoblot analysis shows that the proteins are equally abundant in wild-type and *tsc3*Δ mutant cells (Fig. 4*A*). As mentioned earlier, the mechanism of association of SPT with the membrane is unknown. The predominantly hydrophobic carboxyl-terminal 40 amino acids of Tsc3p suggested that it might serve to anchor SPT to the membrane. However, the membrane association of Lcb1p and Lcb2p is not disrupted in the *tsc3*Δ mutant (Fig. 4*B*). These experiments demonstrate that Tsc3p is not required for the expression or membrane association of Lcb1p and Lcb2p.

To investigate whether Lcb1p and Lcb2p are physically associated and whether their association depends on Tsc3p, immunoprecipitation experiments were conducted. The rabbit polyclonal antibodies to either Lcb1p or Lcb2p coimmunoprecipitate both Lcb1p and Lcb2p, indicating that the two proteins are associated (Fig. 5*A*). A plasmid carrying a GST-tagged allele of *LCB1* (see under "Experimental Procedures") was transformed into the *lcb1*Δ mutant. The Lcb1p-GST fusion protein restores SPT activity (data not shown) and coimmunoprecipitates with Lcb2p (Fig. 5*A, lanes 6* and *7*). Whereas Lcb1p was present in the microsomes from the *lcb2*Δ mutant (Fig. 4), following solubilization of the microsomes and the immunoprecipitation protocol, Lcb1p was not detected in the immunoprecipitates from the *lcb2*Δ mutant microsomes (Fig. 5*A, lanes 8* and *9*). This is due to degradation of Lcb1p without Lcb2p because Lcb1p was not present in the unbound supernatant removed from the protein A-Sepharose beads.

The coimmunoprecipitation of Lcb1p and Lcb2p was not altered in the *tsc3*Δ mutant (Fig. 5*A, lanes 10* and *11*) demonstrating that Tsc3p is not required for the association of Lcb1p with Lcb2p. The coimmunoprecipitation of Lcb1p and Lcb2p was unaffected by the absence of Tsc3p even at 0.6 M NaCl (Fig. 5*B*). Thus, although it is possible that Tsc3p influences the interaction between Lcb1p and Lcb2p, the Tsc3p-independent association of Lcb1p and Lcb2p is stable at high ionic strengths.

Tsc3p Is a Microsomal Protein That Coimmunoprecipitates with Lcb1p and Lcb2p—An epitope-tagged allele of *TSC3* was generated by placing the triple HA epitope between codons 12 and 13 (see under "Experimental Procedures"). The Tsc3p-HA protein is functional because it complements the ts phenotype of the *tsc3*Δ mutant and restores SPT activity. The Tsc3p-HA protein fractionates with the microsomes (Fig. 6*A*) and is solubilized similarly to Lcb1p and Lcb2p with a variety of detergents, demonstrating that Tsc3p is membrane-associated (Fig. 6*B*). The Tsc3p-HA protein has a predicted molecular weight of 13,711, but it migrates with an apparent molecular mass of approximately 18,000 Da (Fig. 6*A*). This may be because Tsc3p-HA has a calculated pI of 5.49. As was mentioned above, proteins with acidic pIs often migrate aberrantly in SDS-PAGE.

The rabbit polyclonal antibodies to either Lcb1p or Lcb2p coimmunoprecipitated Tsc3p-HA along with Lcb1p/Lcb2p (Fig. 6*C*), or conversely, the monoclonal anti-HA antibodies coimmunoprecipitated Lcb1p and Lcb2p along with Tsc3p-HA (Fig. 6*D*). The association of Tsc3p with Lcb1p and Lcb2p is stable at

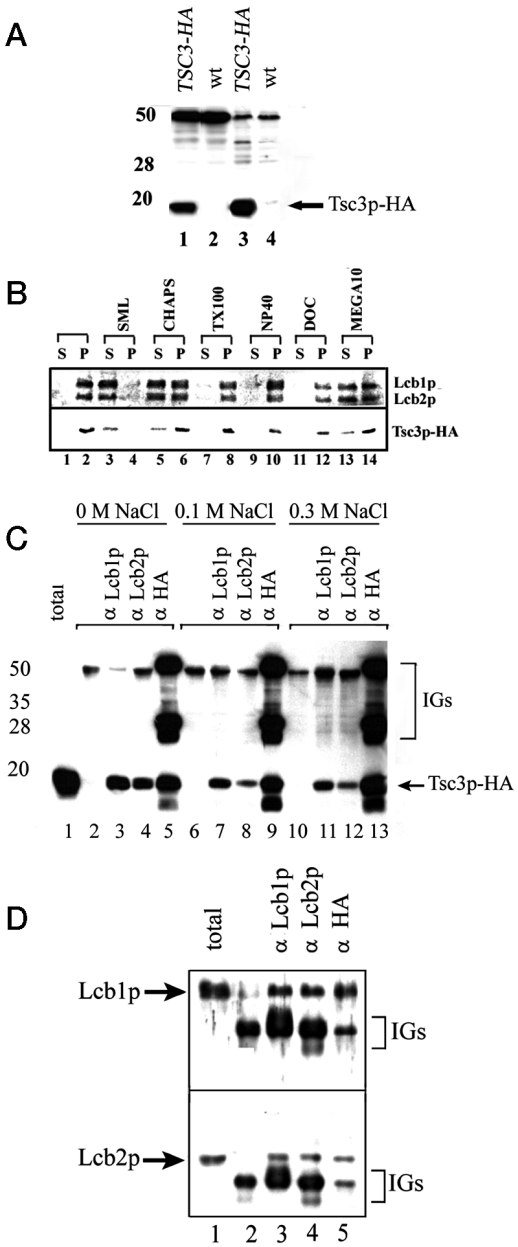

FIG. 6. **Tsc3p is a membrane protein that coimmunoprecipitates with Lcb1p/Lcb2p.** *A,* TDY 2039 cells with (*TSC3-HA, lanes 1* and *3*) or without (*wt, lanes 2* and *4*) a plasmid carrying the *TSC3-HA* allele were used. Proteins from cell lysates (*lanes 1* and *2*) or from microsomes (*lanes 3* and *4*) were separated by 15% SDS-PAGE and immunoblotted with monoclonal anti-HA (12CA5) antibodies. The Tsc3p-HA protein is a microsomal protein. There is an anti-HA cross-reactive protein in the soluble fraction of the cells that migrates near the molecular weight 50,000 marker. *B,* microsomes (100 μl at 1 mg/ml) from cells containing Tsc3p-HA (as in *A*) were incubated for 10 min at 25 °C with the indicated detergents. No detergent was added to the microsomes in the samples in *lanes 1* and *2*. The detergents were used at their critical micelle concentrations: sucrose monolaurate (*SML*) at 2 mM; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (*CHAPS*) at 6 mM; Triton X-100 (*TX100*) at 0.2 mM; tergitol (*NP40*) at 0.06 mM; deoxycholate (*DOC*) at 2 mM, and Mega10 at 6 mM. The samples were centrifuged at $1 \times 10^5 \times g$ for 30 min, and the pellets (*P*) and supernatants (*S*) were subjected to 8% SDS-PAGE for Lcb1p and Lcb2p and 15% SDS-PAGE for Tsc3p-HA. Lcb1p and Lcb2p were detected on the same blot (*top panel*) using the anti-Lcb1p and anti-Lcb2p antibodies as described in the legend to Fig. 4. Tsc3p-HA (*bottom panel*) was detected as in *A*. *C,* the microsomes from the cells containing Tsc3p-HA (*A*) were solubilized with 2 mM sucrose monolaurate as in *B* and centrifuged at $1 \times 10^5 \times g$ for 30 min, and NaCl was added to the supernatant to the indicated concentration. The supernatant was immunoprecipitated using either rabbit preimmune serum (*lanes 2, 6,* and *10*), polyclonal antibodies to Lcb1p (*lanes 3, 7,* and *11*), Lcb2p (*lanes 4, 8,* and *12*), or monoclonal antibodies to HA (*lanes 5, 9,* and *13*). The immunoprecipitated proteins were resolved on 15% SDS-PAGE and immunoblotted with the anti-HA antibodies. The major dark immunoreactive bands in the anti-HA immunoprecipitates are the mouse immunoglobulins. *Lane 1* contains 10 μg of the total microsomal protein. *D,* the immunoprecipitated proteins (same samples as in *C, lanes 2–5*) were resolved by 8% SDS-PAGE and immunoblotted with the Lcb1p (*top panel*) or Lcb2p antibodies (*bottom panel*). *Lane 1* contains 10 μg of total microsomal protein. The dark immunoreactive bands below Lcb1p and Lcb2p are the immunoglobulins (*IGs*).

0.3 M NaCl (Fig. 6*C*). At a higher salt concentration (0.6 M NaCl), Lcb1p and Lcb2p coimmunoprecipitated with each other (Fig. 5*B*), but Tsc3p-HA was not present in the complex (data not shown). These experiments show that Tsc3p physically associates with Lcb1p and/or Lcb2p. It has not been possible to determine whether Tsc3p associates with Lcb1p, Lcb2p, both proteins, or a complex of the two proteins, because of the instability of Lcb2p in the absence of Lcb1p and *vice versa*.

DISCUSSION

Tsc3p is a novel membrane-associated protein that binds to the other proteins required for SPT activity. Tsc3p acts post-translationally and through association with Lcb1p and/or Lcb2p to stimulate the activity of SPT, but it remains to be determined how Tsc3p modulates SPT activity. Although it is not homologous to acyl-CoA carrier proteins, Tsc3p may bind palmitoyl CoA. The other α-oxoamine synthases are soluble proteins that utilize short acyl-CoAs, whereas SPT is membrane-associated and uses palmitoyl-CoA, which itself partitions into the membrane. Therefore, Tsc3p may be required for the appropriate interaction of the acyl CoA substrate with the enzyme. The possibility that Tsc3p is required for the proper interaction of palmitoyl CoA with SPT may provide an interpretation for our data showing that Tsc3p-independent SPT activity is more sensitive to inhibition by palmitoyl CoA. For example, palmitoyl CoA might bind the enzyme nonproductively without Tsc3p. The observation that Lcb1p and Lcb2p are stable and tightly associated in the solubilized microsomes from the *tsc3*Δ mutant suggests that the increased sensitivity to palmitoyl CoA in the absence of Tsc3p is not due to a trivial detergent effect of palmitoyl CoA. The ability to detect and immunoprecipitate Tsc3p will provide the opportunity to investigate whether Tsc3p binds a photoaffinity labeling analog of palmitoyl CoA (22).

Two observations indicate that Tsc3p is required only for interaction with the SPT enzyme. First, addition of LCBs to the growth medium reverses the ts phenotype of the *tsc3*Δ mutant. Second, we have isolated and characterized pseudorevertants of the *tsc3*Δ mutant that can grow at 37 °C.[2] Forty-nine suppressor mutants have been analyzed, and all of them have single amino acid changes at one of eight positions in the *LCB2* gene. The SPT activity is increased 6–10-fold in these *tsc3*Δ mutants as a result of the suppressing mutation in Lcb2p. Determining why the SPT activity in these Lcb2p variants has a reduced dependence on Tsc3p is expected to provide information about the role of Tsc3p. Based on these results, it is concluded that Tsc3p is not required for any essential process besides 3-ketosphinganine synthesis.

Using antibodies generated to either Lcb1p or to Lcb2p for immunoprecipitation experiments, we find that Lcb1p and Lcb2p are membrane-associated proteins that physically associate with one another. This is consistent with the finding of Nishijima and co-workers (21) that antibodies to hamster Lcb2p co-immunoprecipitate both Lcb1p and Lcb2p. We also observe that the level of Lcb2p in microsomes from the *lcb1*Δ

[2] K. Gable, E. Monaghan, and T. M. Dunn, unpublished data.

mutant is greatly reduced, apparently due to instability of Lcb2p in the absence of the interacting Lcb1p. Therefore, the observation that there is no SPT activity detected in microsomes prepared from the *lcb1*Δ mutants of *Saccharomyces cerevisiae* (or in the CHO cell line with a ts mutation in *LCB1* (21)) does not necessarily mean that Lcb1p is required for catalysis. The absence of SPT activity may reflect instability of Lcb2p when Lcb1p is missing. The level of Lcb2p present in the membranes prepared from the CHO mutants that lack detectable Lcb1p has not been reported (21).

SPT catalyzes the committed and apparently rate-limiting step in sphingolipid synthesis, and sphingolipids are essential for the viability of all eukaryotic cells. Although there is a great deal of evidence that the LCBs, and other sphingolipid metabolites, are important signaling molecules, little is known about how SPT activity is regulated. Although we have not identified Tsc3p homologs in the genomes of higher eukaryotic cells, the high degree of sequence conservation between the yeast and mammalian *LCB1* and *LCB2* genes suggests that Tsc3p-like proteins may be required for optimal activity of SPT in mammalian cells as well. Determining how Tsc3p acts to stimulate SPT activity is likely to provide information about the regulation of sphingolipid synthesis.

Mutants in the TSC collection identify many ts alleles of the genes required for synthesis of the long chain bases including the genes for 3-ketosphinganine (*TSC1*/*LCB2*, *TSC2*/*LCB1*, and *TSC3*), dihydrosphingosine (*TSC10*), and phytosphingosine (*TSC7*/*SUR2*) synthesis (10). The *SCS1*/*LCB2* and *SCS2*/*LCB1* genes were also identified in the SCS suppressor screen, but the *scs* mutants were isolated as suppressors of the Ca^{2+} sensitivity of the *csg2*Δ mutant at 37 °C (2), and therefore, the *TSC3* gene (essential at 37 °C) was not represented in the SCS suppressor collection. The screen for LCB-requiring mutants that identified the *LCB1* and *LCB2* genes was conducted at 30 °C (20), and the *tsc3* mutants only display the LCB-requiring phenotype at high temperatures (*e.g.* 37 °C), which probably explains why the *TSC3* gene was not found in that screen. The *tsc3*Δ mutant is viable at 26 °C despite the decreased SPT activity, but the mutant cannot grow at elevated temperatures. This indicates that the requirement for sphingolipids is greater at higher growth temperatures and explains why such a high percentage of the suppressor mutants display the associated ts phenotype.

In addition to the genes required for LCB synthesis, genes required for the synthesis and hydroxylation of the very long chain fatty acids have also been identified through characterization of the TSC suppressor mutant collection. The continued analysis of the *TSC* genes is expected to provide new insights into the regulation of the biosynthetic pathway and into the functions of sphingolipids.

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