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The *Saccharomyces cerevisiae* *TSC10/YBR265w* Gene Encoding 3-Ketosphinganine Reductase Is Identified in a Screen for Temperature-sensitive Suppressors of the Ca^{2+} -sensitive *csg2* Δ Mutant*

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Saccharomyces cerevisiae csg2 Δ mutants accumulate the sphingolipid inositolphosphorylceramide, which renders the cells Ca^{2+} -sensitive. Temperature-sensitive mutations that suppress the Ca^{2+} sensitivity of *csg2* Δ mutants were isolated and characterized to identify genes that encode sphingolipid synthesis enzymes. These temperature-sensitive *csg2* Δ suppressors (*tsc*) fall into 15 complementation groups. The *TSC10/YBR265w* gene was found to encode 3-ketosphinganine reductase, the enzyme that catalyzes the second step in the synthesis of phytosphingosine, the long chain base found in yeast sphingolipids. 3-Ketosphinganine reductase (Tsc10p) is essential for growth in the absence of exogenous dihydro-sphingosine or phytosphingosine. Tsc10p is a member of the short chain dehydrogenase/reductase protein family. The *tsc10* mutants accumulate 3-ketosphinganine and microsomal membranes isolated from *tsc10* mutants have low 3-ketosphinganine reductase activity. His₆-tagged Tsc10p was expressed in *Escherichia coli* and isolated by nickel-nitrilotriacetic acid column chromatography. The recombinant protein catalyzes the NADPH-dependent reduction of 3-ketosphinganine. These data indicate that Tsc10p is necessary and sufficient for catalyzing the NADPH-dependent reduction of 3-ketosphinganine to dihydro-sphingosine.

Sphingolipids are essential components of eukaryotic membranes. They are composed of a polar head attached to a hydrophobic ceramide base. Ceramide contains a fatty acid attached in amide linkage to a long chain base (LCB).¹ Sphingolipid synthesis begins with the formation of the long chain base dihydro-sphingosine (Fig. 1), which requires two enzymes. The first enzyme, serine palmitoyltransferase, catalyzes the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine. This enzyme contains at least two subunits (Lcb1p and Lcb2p) (1, 2, 3). The second enzyme reduces 3-ketosphinganine to dihydro-sphingosine (sphinganine) which is subsequently hydroxylated on C4 by Sur2p forming phytosphingosine (4).

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This manuscript is dedicated to the memory of Troy J. Beeler.

† Deceased June 9, 1998.

¹ The abbreviations used are: LCB, long chain base; IPC, inositolphosphorylceramide; ORF, open reading frame; NTA< nitrilotriacetic acid; PCR, polymerase chain reaction; IPC-C, inositolphosphoryl-(α -hydroxy)phytoceramide.

Phytoceramide is synthesized by the attachment of a very long chain fatty acid to the amine of the LCB. Scs7p hydroxylates the fatty acid moiety to form α -OH-phytoceramide (4, 5). Scs7p hydroxylates the ceramide less efficiently in a *sur2* null mutant (which accumulates dihydroceramide rather than phytoceramide), indicating that hydroxylation of the very long chain fatty acid occurs after formation of ceramide (4).

Aur1p catalyzes the transfer of the phosphoinositol head of phosphatidylinositol to OH-phytoceramide to form inositolphosphorylceramide (IPC-C) in the endoplasmic reticulum (6). In the Golgi, IPC is mannosylated to form mannosylinositolphosphorylceramide (7, 8). This reaction requires two genes, *CSG1* and *CSG2* (1, 9).

The *CSG1* and *CSG2* genes were originally identified in a screen for mutants unable to grow in medium containing 100 mM Ca^{2+} (10). Deletion of either gene causes the accumulation of IPC-C and renders the cells Ca^{2+} -sensitive (1, 9). Mutations that decrease the synthesis of IPC-C or alter its structure suppress the Ca^{2+} sensitivity of *csg1* and *csg2* mutants (1, 4, 5, 9, 11). Therefore, the *csg1* and *csg2* mutants provide a positive selection for sphingolipid synthesis mutants since they are suppressors of the Ca^{2+} -sensitive phenotype.

Many of the genes that can mutate to cause suppression are expected to be essential since sphingolipids are required for viability. Thus, a screen for temperature-sensitive suppressors of Ca^{2+} sensitivity was conducted. Suppressing mutations that confer temperature sensitivity are experimentally powerful for several reasons. 1) They provide a restrictive condition that allows the wild-type gene to be cloned by complementation; 2) analysis of sphingolipid synthesis in the mutants after shifting to the restrictive condition may uncover defects that are not apparent at the permissive temperature; 3) suppressors of the temperature sensitivity are expected to identify new genes involved in sphingolipid synthesis; and 4) finally, the suppressor mutant collection is more likely to identify genes in sphingolipid synthesis that are essential.

In this paper we report the isolation and characterization of temperature-sensitive mutants that suppress the Ca^{2+} -sensitive phenotype of *csg2* mutants and the use of *tsc10* mutants to identify the gene that encodes 3-ketosphinganine reductase.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth—The yeast strains used in this study were TDY2037 (*Mata lys2 ura3-52 trp1* Δ *leu2* Δ), TDY2038 (*Mata lys2 ura3-52 trp1* Δ *leu2* Δ *csg2::LEU2*), TDY2039 (*Mata ade2-101 ura3-52 trp1* Δ *leu2* Δ), TDY2040 (*Mata ade2-101 ura3-52 trp1* Δ *leu2* Δ *csg2::LEU2*), and DBY947 (*Mata ade2-101 ura3-52*). Media were prepared and cells were grown using standard procedures (12). Phytosphingosine and dihydro-sphingosine were purchased from Sigma and added from a 50 mg/ml stock solution (in ethanol) to autoclaved media containing 0.2% tergitol.

Cloning the *TSC10* Gene—Strain LHYa56 (*Mata ade2-101 ura3-52 trp1Δ leu2Δ csg2::LEU2 tsc10-1*) was transformed with a YCp50-based yeast genomic plasmid library (14) selecting for uracil prototrophs. The transformants were replica-plated to YPD and placed at 37 °C, and one transformant had acquired a plasmid that complemented temperature sensitivity. Hybridization studies showed that the complementing genomic fragment resides on ATTC contiguous clone 70147. Subcloning experiments demonstrated that the YBR265W open reading frame was responsible for complementation. Linkage studies confirmed that YBR265W is the ORF that is mutated in the *tsc10* mutants. A 3150-base pair *SalI* fragment extending from a site within YBR263W to a site

within YBR266C was cloned into pRS306 (13). The resulting plasmid was linearized at the *MscI* site in YBR265W and integratively transformed into LHYa56 (*tsc10-1*). The temperature-sensitive phenotype was complemented. Diploids constructed by mating this haploid transformant to a wild-type haploid (DBY 947) were sporulated and dissected. All products of meiosis were temperature-resistant, demonstrating that the YBR265W-carrying plasmid integrated at a site linked to the *tsc10-1* locus.

Construction of the *tsc10::TRP1* Null Mutant Allele—The 3150-base pair *SalI* fragment carrying the YBR265W ORF was cloned into pUC19 and a blunt-ended PCR fragment carrying the *TRP1* gene was ligated into the *MscI* site in codon 252 of *YBR265w/TSC10*. The disrupted allele, liberated with *SalI*, was transformed into a diploid (formed by crossing TDY2038 with TDY2040), and tryptophan prototrophs were selected (15). Sporulation and dissection of the diploid demonstrated that the *TSC10* gene is essential for viability.

Analysis of Long Chain Base—Fifty A_{600} units of cells (A_{600} of 0.5–1.0) were pelleted, washed in water, and resuspended by vortexing in 0.2 ml of 0.5 M NH_4OH . LCBs were extracted by a modification of the method of Williams *et al.* (16). Two ml of $\text{CHCl}_3\text{:MeOH}$ (1:2) and glass beads (0.5 volume) were added, and the cells were vortexed for 1 min and sonicated for 15 min in a Branson 42 bath sonicator. The supernatant was removed to a fresh tube, and 1 ml of CHCl_3 , 2 ml of 0.5 M NH_4OH , and 0.02 ml of 3 M KCl were added with vortexing after each addition. The bottom layer was washed twice with 2 ml of 30 mM KCl and dried under N_2 . Extract corresponding to 10 A_{600} units of cells was spotted on a silica gel TLC plate, which was developed using $\text{CHCl}_3\text{:MeOH:2 M NH}_4\text{OH}$ (40:10:1) as solvent (17). Phytosphingosine, dihydrosphingosine and sphingosine standards were purchased from Sigma. The 3-ketosphingosine standard was custom-synthesized by Matreya, Inc. (Pleasant Gap, PA). LCBs were visualized by spraying the plates with 0.2% ninhydrin in ethanol and heating at 180 °C for 10 min.

NaBH_4 Reduction of Long Chain Bases—The long chain base extract (from 10 A_{600} units of cells) or the 3-ketosphingosine standard (50 μg) was dried and resuspended in 200 μl of 95% ethanol. NaBH_4 (0.2 g) was added, and the sample was incubated at room temperature for 20 min. Hydrochloric acid (100 μl of 0.1 N) was added followed after 5 min by NH_4OH (100 μl of 2 M), and LCBs were re-extracted as described above.

Membrane Preparation—Exponentially growing cells (750 A_{600} units) were harvested and washed in water. The cells were resuspended at 2 g/ml by vortexing in 50 mM Tris (7.5), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM β -mercaptoethanol (TEPM buffer). Glass beads were added to the meniscus, and the cell walls were disrupted by vortexing eight times for 30 s with cooling on ice between vortexing. The homogenate was transferred to a centrifuge tube, and the glass beads were washed with TEPM buffer until the wash was clear. The washes were pooled and centrifuged at 4000 $\times g$ for 10 min. The supernatant was centrifuged at 40,000 $\times g$ for 40 min. The pellet was resuspended in the TEPM buffer using a Dounce homogenizer and centrifuged at 40,000 $\times g$ for 40 min. This final pellet was resuspended in TEPM +

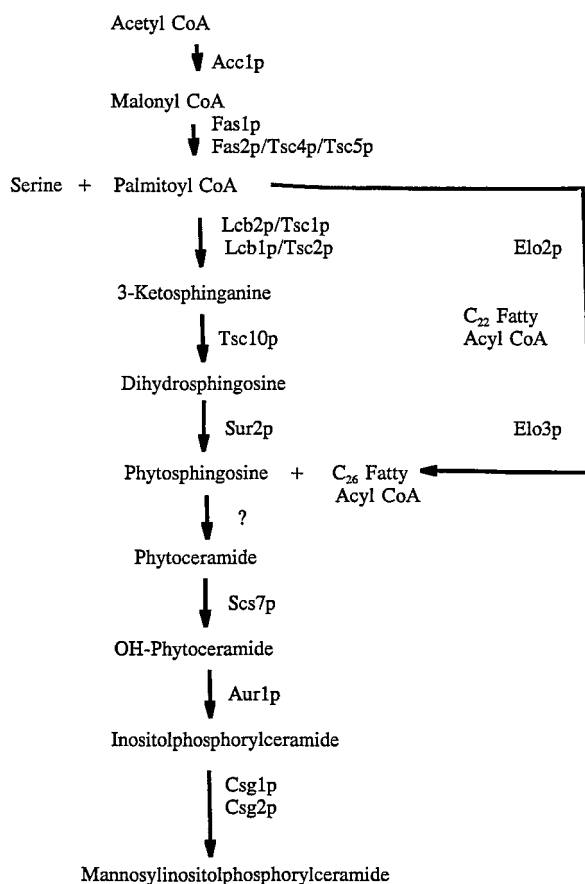


FIG. 1. Synthesis of mannosylinositol phosphorylceramide from acetyl CoA.

TABLE I
The *TSC* mutant collection

Mutants were placed in complementation groups by mating the *Mata tsc* mutant isolates with the *Mata tsc* isolates. In the cases where only one representative was found, the mutant was back-crossed to wild-type and *tsc* haploids in the other mating type were obtained for complementation testing. The suppressors were isolated on YPD + 20 mM Ca^{2+} with (CJY isolates) or without (LHY isolates) 80 μM bathocuproine disulfonate. The gene that is mutated in the isolates of each complementation is listed according to its nomenclature in the *Saccharomyces* Genome Database.

TSC group	Strains	Mutated gene
TSC1	LHYa38, a64, a67, a137, a21, a45, a47, a121, a127, a155, a163	LCB2/SCS1
TSC2	CJYa5, a19, a1, a2, a24	LCB1
	LHYa22, a24, a26, a81	
	CJYa3, a4, a7, a12, a14, a31	
TSC3	LHYa60	
	CJYa16, a18, a8, a11	
TSC4	CJYa6	FAS2
TSC5	CJYa30	FAS2
TSC7	LHYa11, a28, a105, a34	SUR2
TSC8	LHYa33, a37, a33, a111, a116, a157, a166	SIN3
TSC9	LHYa39, a4, a38, a43, a61, a101, a137, a141	RPD3
TSC10	LHYa56, 108, 109	YBR265w
TSC11	LHYa53	YER093c
TSC13	LHYa15	YDL015c
TSC14	LHYa82	TOR2
TSC15	LHYa86	MSS4

FIG. 2. Alignment of the amino acid sequence of Tsc10p with representative members of the short chain dehydrogenase/reductase enzyme family. The sequences of Tsc10p (TSC), human FVT protein (FVT), *Streptomyces* 20- β -hydroxysteroid dehydrogenase (BHD), and human sepiapterin reductase (SPR) are compared. The secondary structure prediction (H, α -helix; S, β -sheet) is taken from the Munich Information Center for Protein Sequences and was obtained using the program Predator (25). The assignment of the α -helices and β -sheets in Tsc10p agrees with those assigned to *Streptomyces* 20- β -hydroxysteroid dehydrogenase and human sepiapterin reductase by Jornvall *et al.* (18). The amino acids that are conserved in all four proteins are indicated below the alignment. The lysine and the tyrosine present in the catalytic center of all the short chain dehydrogenase/reductase enzymes as well as the highly conserved Ser that is usually located 13 residues before the Tyr are indicated by asterisks. The hydrophobic tail of Tsc10p is underlined.

FVT	1	MLLLAAAFVAVFVLLLYMVSPLISP
		β A α B β B SSSSS HHHHHHHHHHHHHH SSSSSSS HHHHHHHHHH
TSC	1	<u>MKFTLEEDQVLLITGGSSOGLGKEFAKKYVNEAENTKIIIVSRSEARH</u> .LDTCNEL
FVT	26	<u>KPLAEPGAHVVTGGSSGIGKCIATIECYKQG</u> ..AFITLVARNEDKL.LQAKKEI
BHD	1	<u>MNDHSGKTVIITGGGLGAEARQVAAGARVVLADV</u> ...LDEGA.....
SPR	1	<u>MEGGEGRAVCLLTGASRGFGRTLAPLLASLLSPGSVL</u> .WLSARNDEALR.....
		L T G G
		α C β C α D HHHHHHHHH HHHHHH HHHHHH SSSHHHHHHHHHHH
TSC	54	<u>RIEAHRRRETDEGOVQHKLAAPLDLEQLFVYPCDLSVCSVECLFNALRDL</u>
FVT	77	<u>EMHS</u> .INDK....QV...ICISVDVSOD.....YQVENVIKQAE.K
BHD	45	<u>ATARELG</u>DAARYOHLVDVTIREDWQR...VVAYA
SPR	49	<u>QLEAEI</u> GAERSG.....LRVVRVPADLGAAGLQQLLALREL
		β D α E SSS SSS HHHHHHHHHHHH
TSC	108	<u>I</u> ...LQ...TLC.CAGGAVPKLFRGLSGHELNLGMDINXKTTLNVAHQIALAEQT
FVT	112	<u>I</u> ...GPVDMVNCAGMAVSGKFEDEVSTFERLMSINYLGSVYPSRAVITTMKE
BHD	75	<u>REEFGSVDGLVNNAGLSTGMFLETES</u> VERFRKVVLDINLTGVFIMGKTVIPAMKD
SPR	88	<u>RPKGLQRLLIN</u> AGSLGDVSKGFV...DLS DSTQVNNY WALNLT SMLCLTSSVL
		L A G N
		β E α F HH HSSSSSSS HHHHHHHHHHHHHHHHHH HS
TSC	158	<u>KE</u>HHLTIE.SSATALYDFVGYSOYAPAKAAIKSIIVAILRQELT..NF
FVT	163RRVGRIVFVSSQAGQLGLFGFTAKSASKFAIRGIAEALQMEVVKPYNV
BHD	129AGGSTVNISSAAGLMGLALTSSYGASKWGVRGISKLAAVEIGTDRI
SPR	140	<u>KAFPDP</u> PGLNRTTVNIISSLCALQPEKGWALY CAGKAARDMEFOVLALEP..NV
		SS Y K L E * * *
		β F α G SSSSS HHH HHHHHHHHHHHH
TSC	202	<u>RTSCV</u> YPG.NFESEGETVEQ...LTKPEITKLEGGPSDAIPCKQRCDI AKSE
FVT	210	<u>YITVA</u> YPP.D'DTTPGAEEN...RHKPLETRFISETTSVCKPEQVAKQIVKDA
BHD	176	<u>RVNSV</u> HPG.MTYTPMTAETG...RQGGGNYPNTPMGRVGEGETAGA.VVKLI
SPR	191	<u>RVLNYA</u> PGPLDMDQQLARETSVDPDMRKGQLQELKAKGKLVDC KVSAQKLLSL
		P
		H HHHHHHHHHH SSSSSSSSS H
TSC	251	<u>ARGDEDV</u> FTDFVGMIMGMDLGLTAKKSRFVPELWIFGVL SNILVVPFVMGCS
FVT	259	<u>IQGNF</u> NSLGS GYML SALTCGMAPVTSITEGEGQVVMTGLFRTIAL EYLG SFD
BHD	226	<u>SDTSS</u> YVTGAELAVDGGWTTGPTVKYYMGQ 255
SPR	245	<u>EKDEFK</u> .SGA HVDF YDK 261
		HHHHHHHH
TSC	305	<u>WYIRK</u> WFRENDGKKAN 320
FVT	313	<u>SIVR</u> RCMMQREKSENADKTA 332

TSC = *S. cerevisiae* Tsc10p

FVT = Human FVT protein

BHD = *Streptomyces* 20- β -hydroxysteroid dehydrogenase

SPR = Human Sepiapterin reductase

33% glycerol at a protein concentration of about 12 mg/ml and stored at -80°C .

Assay of Serine Palmitoyltransferase and 3-Ketosphinganine Reductase—The reaction was initiated by the addition of 1 mg of protein and 60 μmol of palmitoyl CoA to a 300- μl reaction mixture containing 0.1 M Hepes (8.3), 5 mM dithiothreitol, 2.5 mM EDTA, 1 mM serine (10 $\mu\text{Ci/ml}$ [^3H]serine), 50 μM pyridoxal 5'-phosphate (16). In some samples, an NADPH-regenerating system was added (2.4 mM glucose 6-phosphate, 2–8 units of glucose-6-phosphate dehydrogenase, 1 mM NADPH) to allow reduction of the 3-ketosphinganine intermediate. After 10 min at 37°C , the reaction was terminated by addition of NH_4OH to 0.25 M. The LCB was extracted by successively adding 1.5 ml of $\text{CHCl}_3\text{:MeOH}$ (1:2), 30 μg of carrier dihydrosphingosine, 1 ml of CHCl_3 , 2 ml of 0.5 M NH_4OH with vortexing after each addition. The sample was centrifuged, and the upper aqueous layer was removed. The lower phase was washed two times with 2 ml of 30 mM KCl. The extracted LCBs were dried and run on a silica gel TLC plate developed with $\text{CHCl}_3\text{:MeOH}$:2 M NH_4OH (40:10:1).

Expression of Tsc10p in *Escherichia coli*—The TSC10 ORF was subcloned into the pBAD/Myc-His B plasmid (Invitrogen) resulting in arabinose-inducible expression of Tsc10p with a Myc and polyhistidine tag at the COOH terminus. A construct having the full-length Tsc10p, as well as one that lacks the COOH-terminal 38 amino acids, was made. The PCR primers used to amplify the Tsc10-containing fragments were 5'-GGCCCCATGGAGTTTACGTTAGAAGACCAAGTTGTG-3' (the NH_2 -terminal primer for both fragments), 5'-GGCCTCTAGATTGTTGGCCTTCTTGCCGTCATTTTCAC-3' (the COOH-terminal primer for the full-length Tsc10p construct), and 5'-GGCCTCTAGATTGCGAACAAAGCGCTTTTCTTTGCGG-3' (the COOH-terminal primer for the construct lacking the COOH-terminal 38 amino acids). The PCR-generated fragments were digested with *Nco*I and *Xba*I (sites shown in bold on the PCR primers) and ligated into pBAD-Myc-His-B. The underlined ATG in the *Nco*I site is the start codon, and codon 2 is changed from lysine to glutamate in the recombinant protein. The plasmids were transformed into TOP10 cells (Invitrogen).

Purification of the Recombinant Myc-His-tagged Tsc10 Proteins—

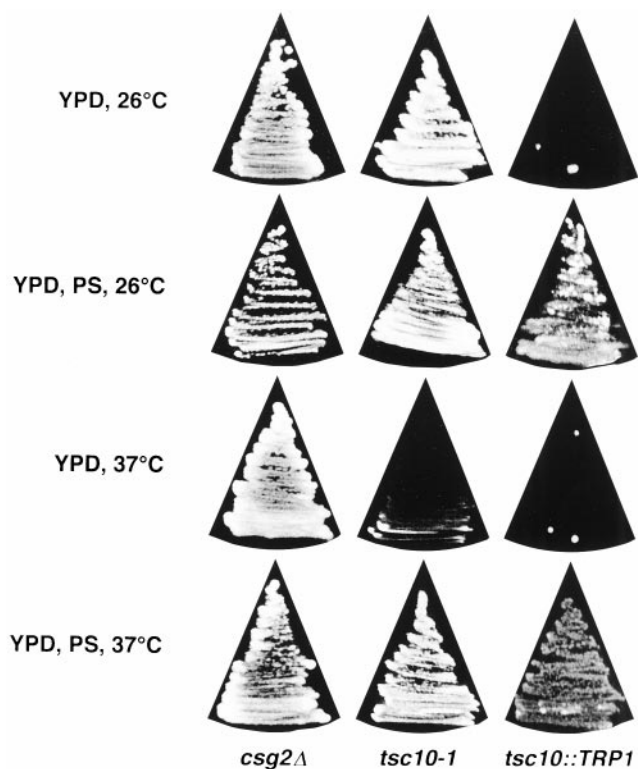


FIG. 3. The temperature sensitivity of the *tsc10* mutants is rescued by phytosphingosine. TDY2040 (*csg2Δ*), TDY2040 with the *tsc10* gene knocked out (*tsc10::TRP1*) and the temperature-sensitive mutant LHYa56 (*tsc10-1*) were streaked onto YPD \pm 20 μ M phytosphingosine (PS) and incubated at 26 °C or 37 °C for 2 days.

The transformed cells were grown in LB + ampicillin to an A_{600} of 0.8. Cells were pelleted and resuspended (A_{600} of 4) in M10 medium with 0.5% arabinose for 2 h. Harvested cells were resuspended in 4 ml (100 A_{600} /ml) of 50 mM Tris (pH 7), 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and pepstatin A, 1 mg/ml lysozyme, and 0.1 mg/ml DNase I. After several cycles of freeze/thaw, the lysed cells were centrifuged at 10,000 \times g for 10 min. The supernatant was adjusted to 300 mM NaCl and 1 mM imidazole, and 2 ml of a 50% slurry of Ni-NTA agarose (Qiagen) was added for 1 h at 4 °C to allow the polyhistidine-tagged protein to bind. The resin was washed with 20 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate (pH 7), and eluted with 250 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate. The purification was followed by SDS-PAGE. The gels were either Coomassie-stained or transferred to nitrocellulose for Western blot analysis. Anti-Myc horseradish peroxidase-conjugated antibodies (Invitrogen), which recognize the Myc epitope on the recombinant proteins, were detected using the ECL system (Amersham Pharmacia Biotech).

Assay of the Recombinant Tsc10 Proteins—The activity of the recombinant Tsc10 proteins was measured in 50 mM Hepes (6.9), 133 μ M NADPH, 0.2% tergitol, 80 μ M 3-ketosphinganine at 25 °C either by thin layer chromatography analysis to follow the conversion of 3-ketosphinganine to dihydrosphingosine, or by monitoring the decrease in NADPH absorbance at 340 nm.

RESULTS

Isolation of Mutants That Display Both Suppression of Ca^{2+} Sensitivity of *csg2Δ* Mutants and Temperature Sensitivity—Suppressors of the Ca^{2+} -sensitive phenotype of *csg2* mutants were isolated by streaking *csg2Δ* (null) mutant cells (TDY 2038 and TDY 2040) onto YPD plates containing 20 mM $CaCl_2$ with (the CJY mutant strains) or without (the LHY mutant strains) 80 μ M bathocuproine disulfonate at 26 °C. Bathocuproine disulfonate is a copper chelator; it inhibits hydroxylation of IPC-C thereby increasing the Ca^{2+} sensitivity of *csg2Δ* mutant cells (9). Suppressors were tested for ability to grow on YPD plates at 37 °C, a permissive condition for the starting *csg2Δ* strains. The goal was to obtain mutants in which the same mutation

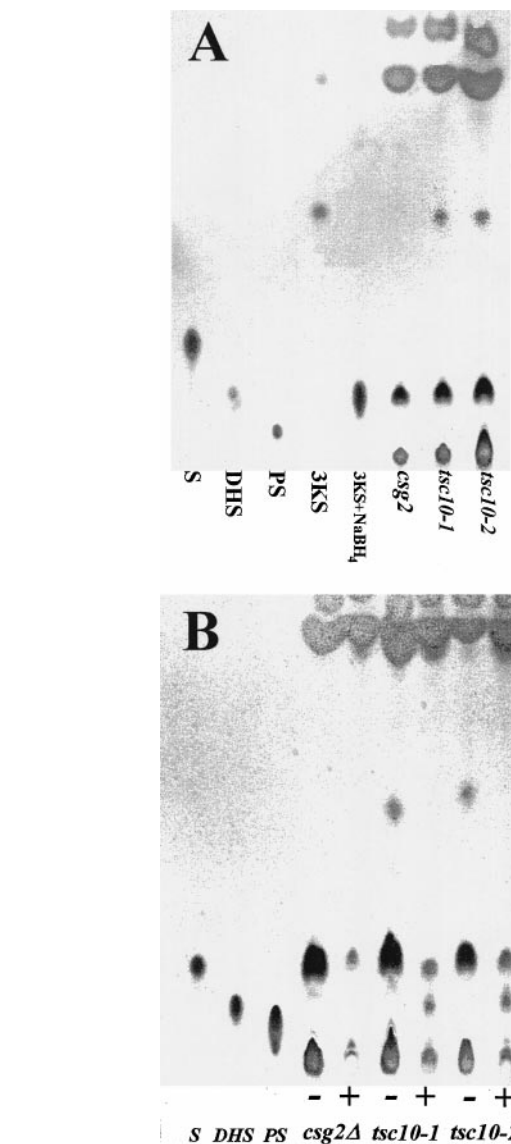


FIG. 4. The *tsc10* mutants accumulate 3-ketosphinganine. LCBs were extracted from TDY2040 (*csg2Δ*), LHYa56 (*tsc10-1*), and LHYa108 (*tsc10-2*) cells grown in YPD medium at 26 °C and analyzed by thin layer chromatography as described under “Experimental Procedures.” A, the *tsc10* mutants (lanes 7 and 8) accumulate a ninhydrin-reactive lipid with the same mobility as 3-ketosphinganine (3KS). The 3-ketosphinganine standard (lane 4) is reduced by treatment with NaBH₄ to dihydrosphingosine (DHS) (lane 5). B, LCBs extracted from TDY2040 (*csg2Δ*), LHYa56 (*tsc10-1*), and LHYa108 (*tsc10-2*) were subjected to thin layer chromatography analysis before (–) or after (+) treatment with NaBH₄. The lipid that accumulates in the *tsc10* mutants is reduced to a species that migrates with dihydrosphingosine (DHS) by NaBH₄ reduction. The identity of the species that migrates between phytosphingosine (PS) and dihydrosphingosine (DHS) in A, and with sphingosine (S) in B is unknown.

that causes suppression of Ca^{2+} sensitivity simultaneously confers temperature sensitivity.

In a screen of 946 suppressors, 59 were found to have a recessive temperature-sensitive phenotype. Complementation of the mutations was determined by testing the temperature sensitivity of diploids constructed by pair-wise mating of the *tsc* haploid mutant strains of opposite mating types. As shown in Table I, the 59 mutants fall into 15 complementation groups. These groups are named TSC for temperature-sensitive suppressors of *csg2* mutants. With one exception (TSC6), tetrad analysis of members from each complementation group confirmed that the temperature-sensitive and suppressing muta-

tions are genetically linked, indicating that suppression and temperature sensitivity arose from a single mutation (data not shown). In the case of the *TSC6* complementation group (which consists of a single representative), the suppressing mutation is in the *SCS7* gene (1, 4, 5) and the temperature-sensitive mutation is unlinked. The identities of several of the *TSC* genes have been determined and are listed in Table I.

***TSC10* Encodes 3-Ketosphinganine Reductase**—The *TSC10* gene was cloned by complementation of the temperature-sensitive phenotype of the LHYa56 (*tsc10-1*) mutant as described under "Experimental Procedures." The complementing gene was found to reside on chromosome II between ORF YBR261c and YBR270c. Subcloning identified the complementing ORF as YBR265w. An integrating plasmid carrying the YBR265w ORF was integratively transformed into the *tsc10-1* mutant, which resulted in complementation of the temperature-sensitive phenotype. When this transformant was crossed to a wild-type (*TSC10*) haploid and the diploids were sporulated and dissected, all products of meiosis were temperature-resistant, indicating that YBR265w is the wild-type *TSC10* gene.

Comparison of the sequence of Tsc10p with proteins in GenBank identifies Tsc10p as a member of the short chain dehydrogenase/reductase protein family (Fig. 2). This family includes over 60 enzymes found in both prokaryotic and eukaryotic cells. Although these enzymes display only 15–30% sequence identity, the family is characterized by a YXXXK sequence (X = any amino acid) found in the catalytic site (18). In many cases, a serine lies 13 residues amino-terminal to the conserved tyrosine. These three amino acids are believed to participate in catalysis by facilitating transfer of a proton from tyrosine to the substrate (19). In Tsc10p, the serine is at position 167, and the tyrosine and lysine residues are amino acids 180 and 184. The short chain dehydrogenase/reductase enzymes also contain a conserved sequence, GXXXGXXG (amino acids 14–20 of Tsc10p), which forms a turn between a β -strand and an α -helix that borders the NADPH-binding domain known as the Rossmann fold (19, 20).

There is a stretch of 28 non-charged, mostly hydrophobic amino acid residues (280–307) close to the COOH terminus (320), which is not predicted to be part of the catalytic domain

(1 to about 250) based on homology to other members of the short chain dehydrogenase/reductase family (Fig. 2). Perhaps the hydrophobic domain functions to anchor 3-ketosphinganine reductase to the endoplasmic reticulum membrane. The di-lysine motif at position -3 and -4 from the COOH terminus may function to localize Tsc10p to the endoplasmic retention (21) (Fig. 2).

Since serine palmitoyltransferase deficiency suppresses the Ca^{2+} -sensitive phenotype of *csg2Δ* mutants, it was believed that a deficiency in the next enzyme in the pathway, 3-ketosphinganine reductase, might also suppress. Tsc10p, a member

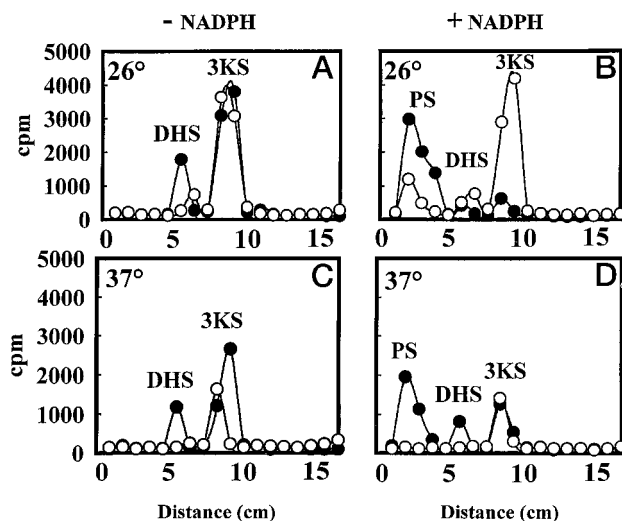


FIG. 5. The *tsc10* mutants are deficient in 3-ketosphinganine reductase activity. Membranes were prepared and serine palmitoyltransferase and 3-ketosphinganine reductase were assayed as described under "Experimental Procedures." TDY2040 (●) and LHYa56 (*tsc10-1*) (○) cells were grown in YPD at 26 °C (A and B) or at 26 °C and then shifted to 37 °C for 3 h (C and D) prior to membrane preparation. Following a 10-min incubation, the LCBs were extracted and separated by thin layer chromatography. Each lane of the TLC was divided into 20 equal fractions, and the silica gel was removed and counted. The position of migration of the 3-ketosphinganine (3KS), dihydrosphinganine (DHS), or phytosphinganine (PS) standards is indicated.

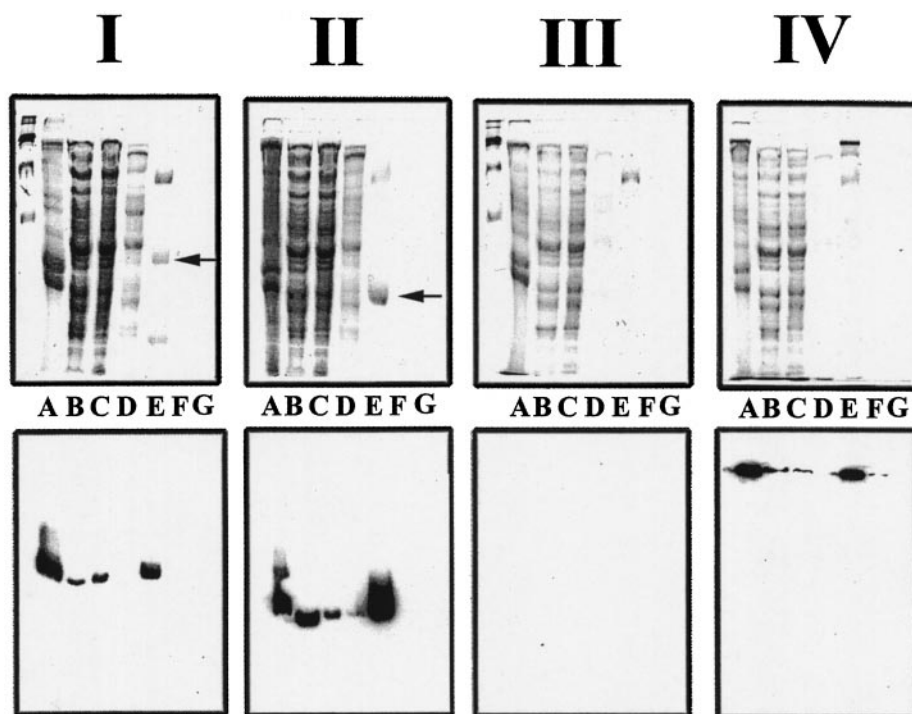


FIG. 6. Isolation of recombinant Myc-His₆-tagged Tsc10p by Ni-NTA chromatography. Lysates of *E. coli* Top10 (Invitrogen) cells expressing full-length Tsc10p-Myc-His₆ (I), the truncated Tsc10p-Myc-His₆ (II), no recombinant protein (III), or LacZ-Myc-His₆ (IV) were passed through a Ni-NTA column. Fractions of the pellet (A), lysate (B), flow-through (C), wash (D), and eluants (E–G) were analyzed by SDS-PAGE. Gels were Coomassie-stained (top row) or transferred to nitrocellulose for Western blot analysis using anti-Myc antibodies (bottom row). The arrows indicate the recombinant full-length (I) and truncated (II) Tsc10p proteins.

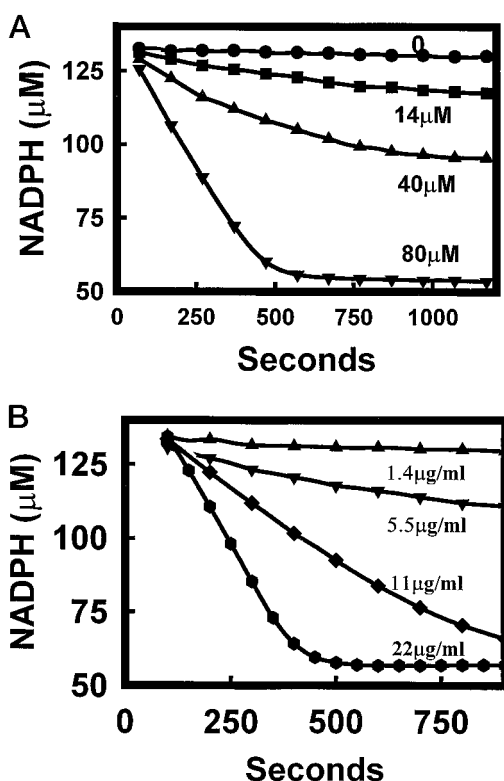


FIG. 7. Recombinant Tsc10p catalyzes the 3-ketosphinganine-dependent oxidation of NADPH. *A*, partially purified recombinant truncated Tsc10-Myc-His₆ protein (10 μg) was added to a cuvette containing varying amounts of 3-ketosphinganine (as indicated) in 750 μl of the standard reaction buffer (see "Experimental Procedures") and the oxidation of NADPH was monitored by measuring the decreased absorbance at 340 nm. *B*, varying amounts of the partially purified recombinant truncated Tsc10-Myc-His₆ protein were added to the standard reaction containing 80 μM 3-ketosphinganine and the reaction was measured as in *A*.

of the short chain dehydrogenase/reductase family of enzymes that reduce ketones to hydroxyl groups, was considered a good candidate for 3-ketosphinganine reductase. Mutants in 3-ketosphinganine reductase are predicted to be rescued by the addition of either dihydrosphingosine or phytosphingosine to the growth medium. Indeed, the temperature-sensitive phenotype of the *tsc10* mutants is reversed by exogenous phytosphingosine (Fig. 3) and dihydrosphingosine (data not shown). In contrast, 3-ketosphinganine, which bypasses the requirement for serine palmitoyltransferase (22), does not rescue the *tsc10* mutants. This is consistent with *tsc10* mutants having a 3-ketosphinganine reductase deficiency.

The genomic *TSC10* gene was disrupted and the locus marked with a *TRP1* gene as described under "Experimental Procedures." The *tsc10Δ* mutants are inviable in the absence of the plasmid-borne *TSC10* gene unless the growth medium contains phytosphingosine or dihydrosphingosine (data not shown and Fig. 3).

Normally 3-ketosphinganine does not accumulate to levels that are detectable by ninhydrin staining of lipid extracts analyzed by thin layer chromatography. However, the *tsc10* mutant strains accumulate a ninhydrin-reactive lipid that has the same mobility as 3-ketosphinganine (Fig. 4A). The sphingosine, dihydrosphingosine, and phytosphingosine standards react with ninhydrin to form a purple-colored product typical of the adduct formed with primary amines. In contrast, the 3-ketosphinganine standard and the lipid that accumulates in the *tsc10* mutants forms a brown-colored product. 3-Ketosphinganine reacts with sodium borohydride to produce dihydrosphing-

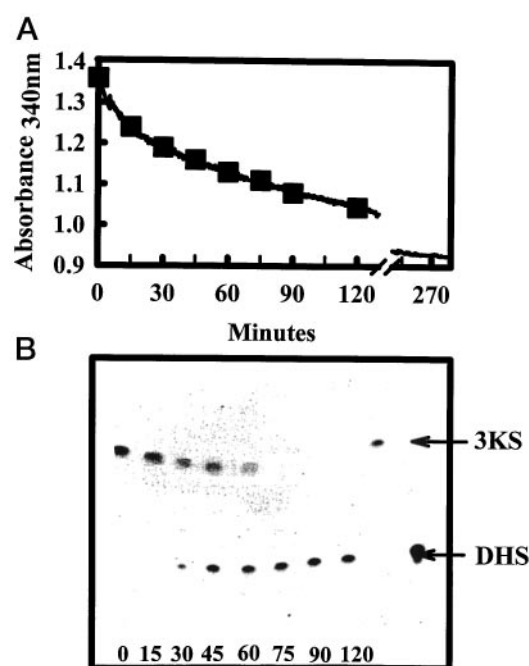


FIG. 8. Recombinant Tsc10p catalyzes the NADPH-dependent reduction of 3-ketosphinganine to dihydrosphingosine. The reaction conditions are similar to those described for Fig. 7 except tirtol was omitted. The reaction was monitored by following the decrease in absorbance of NADPH at 340 nm (*A*). At the indicated times (0, 15 min, etc.), 0.5-ml aliquots were removed and long chain base was extracted and analyzed by thin layer chromatography (see "Experimental Procedures") along with 3-ketosphinganine (3KS) and dihydrosphingosine (DHS) standards (*B*).

gosine (Fig. 4A). The ninhydrin-reactive lipid that accumulates in the *tsc10* mutants is also reduced to a lipid with the same mobility as dihydrosphingosine (Fig. 4B).

3-Ketosphinganine synthesis is catalyzed by serine palmitoyltransferase. Introduction of a temperature-sensitive allele of the *TSC1/LCB2* gene into a *tsc10* mutant to generate a *tsc1tsc10* double mutant blocks accumulation of the ninhydrin-reactive lipid (data not shown). Based upon its mobility on thin layer chromatography, its color after reaction with ninhydrin, its reduction by NaBH₄, and its dependence on serine palmitoyltransferase for accumulation, it is concluded that this lipid is 3-ketosphinganine, consistent with Tsc10p encoding 3-ketosphinganine reductase. The activity of 3-ketosphinganine reductase in microsomal membranes isolated from wild-type and *tsc10* mutants was compared. Wild-type microsomes contain both serine palmitoyltransferase and 3-ketosphinganine reductase activities (16, 23, 24). Serine palmitoyltransferase catalyzes the formation of [³H]3-ketosphinganine from palmitoyl-CoA and [³H]serine (Fig. 5A). Microsomes from wild-type and *tsc10-1* mutant cells have similar serine palmitoyltransferase activity. In the presence of NADPH and wild-type microsomes, [³H]3-ketosphinganine is reduced to [³H]dihydrosphingosine, which is subsequently hydroxylated to [³H]phytosphingosine (Fig. 5, *B* and *D*). In contrast, microsomes prepared from the *tsc10-1* mutant cells after a 3-h shift to 37 °C display no 3-ketosphinganine reductase activity (Fig. 5D). Even microsomes prepared from the *tsc10-1* mutant cells maintained at 26 °C display reduced 3-ketosphinganine reductase activity (Fig. 5B). This experiment demonstrates that Tsc10p is required for 3-ketosphinganine reductase activity.

To confirm that Tsc10p catalyzes 3-ketosphinganine reduction, Tsc10p containing a Myc epitope and a His₆ domain fused at its COOH terminus was expressed in *E. coli* under control of the arabinose-inducible pBAD promoter, isolated using Ni-

NTA chromatography, and assayed for reductase activity. Two constructs were made; in one, the Myc and polyhistidine tags followed the last amino acid of the full-length protein. In the other, the carboxyl-terminal 38 amino acids (containing the potential membrane anchoring domain) of Tsc10p were omitted. As shown in Fig. 6, both tagged proteins were partially purified by Ni-NTA chromatography.

The full-length and the carboxy-truncated purified proteins catalyze a 3-ketosphinganine-dependent oxidation of NADPH to NADP (Fig. 7). NADH does not substitute for NADPH in the reduction. The long chain bases extracted from aliquots of the reaction were analyzed by thin layer chromatography. 3-Ketosphinganine is converted to dihydrosphingosine (Fig. 8B) in a reaction that depends on the addition of NADPH confirming that the recombinant protein is 3-ketosphinganine reductase. The reaction is stimulated 10-fold by 0.2% tertitol, but in this reaction (Fig. 8) detergents were omitted since they interfere with the long chain base extraction and the separation on thin layer chromatography. These experiments also demonstrate that the carboxyl-terminal 38 amino acids are not required for enzymatic activity.

DISCUSSION

Mutations that decrease the rate of IPC-C synthesis or alter its structure suppress the Ca^{2+} -sensitive phenotype of *csg2Δ* mutants (1, 4, 5, 9, 11). Since IPC is essential for growth, it was expected that a temperature-sensitive suppressor collection would identify essential genes required for IPC synthesis. This expectation is confirmed by the finding that temperature-sensitive alleles of the serine palmitoyltransferase genes (*TSC1* and *TSC2*) are among the most represented members of the collection.

The *TSC10* gene was cloned, and its sequence identified it as a member of the short chain reductase/dehydrogenase enzyme family. The results presented in this report demonstrate that Tsc10p is the enzyme that converts 3-ketosphinganine to dihydrosphingosine. Although the enzymes in this family show low sequence identity and varied substrates, their well conserved secondary structures show they possess a single domain comprising the cofactor-binding and active sites. Tsc10p has a stretch of 28 hydrophobic amino acids (residues 280–307) at its carboxyl terminus, which could anchor the protein to the membrane. There are 13 additional amino acids including a KK motif located at position –3 and –4 from the end of the protein. In some cases, dilysine motifs at the carboxyl terminus specify retention of the protein within the endoplasmic reticulum (21). Tsc10p, which fractionates with the microsomes, is expected to

be an endoplasmic reticulum protein since that is the location of IPC synthesis. These amino acids are not required for enzymatic activity.

Based on our analysis of the *TSC* genes, it is expected that several other genes required for sphingolipid synthesis will be identified from the continued study of the temperature-sensitive suppressors. Many of the complementation groups are represented by a single mutant isolate, making it clear that the collection is far from saturated. This work promises to provide new information about the enzymes required for sphingolipid synthesis, regulation of the biosynthetic pathway, and functions of sphingolipids.

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