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Hydroxylation of *Saccharomyces cerevisiae* Ceramides Requires Sur2p and Scs7p*

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The *Saccharomyces cerevisiae SCS7* and *SUR2* genes are members of a gene family that encodes enzymes that desaturate or hydroxylate lipids. Sur2p is required for the hydroxylation of C-4 of the sphingoid moiety of ceramide, and Scs7p is required for the hydroxylation of the very long chain fatty acid. Neither *SCS7* nor *SUR2* are essential for growth, and lack of the Scs7p- or Sur2p-dependent hydroxylation does not prevent the synthesis of mannosyldiinositolphosphorylceramide, the mature sphingolipid found in yeast. Deletion of either gene suppresses the Ca^{2+}-sensitive phenotype of *csg2*Δ mutants, which arises from overaccumulation of inositolphosphorylceramide due to a defect in sphingolipid mannosylation. Characterization of *scs7* and *sur2* mutants is expected to provide insight into the function of ceramide hydroxylation.

Sphingolipids, essential components of eukaryotic plasma membranes, consist of a hydrophilic head attached to a ceramide. Ceramides contain a fatty acid attached to a sphingoid base through an amide linkage (Fig. 1). They can be classified according to their level of hydroxylation (1); both the sphingoid and the fatty acid moieties are found with different levels of hydroxylation (Fig. 1). In mammals, the sphingoid moiety is mostly sphingosine, which is desaturated at C-4,5; however, some is phytosphingosine that is hydroxylated at C-4, or dihydrosphingosine, which is neither desaturated at C-4,5 nor hydroxylated at C-4 (2, 3). In the yeast *Saccharomyces cerevisiae*, the C-4 is mostly hydroxylated (1). The fatty acid that is attached to the sphingoid base is either un-, mono-, or dihydroxylated (1). In yeast, the first hydroxylation of the fatty acid moiety occurs in the endoplasmic reticulum, and the second hydroxylation is in the Golgi apparatus (4) and requires Cu^{2+} and the Golgi copper transporter encoded by *CCC2* (5).

The physiological role of the different hydroxylation states is not known. Hydroxylation of ceramide and sphingolipids may alter their cellular location, their effect on the physical properties of membranes, and their interaction with proteins either as a substrate or regulator. Identification of the genes and proteins required for the hydroxylation reactions will facilitate the investigation of the function of the hydroxyl groups.

The *S. cerevisiae* protein Scs7p is required for the first hydroxylation of the ceramide fatty acid moiety (6). This enzyme belongs to a family of desaturase/hydroxylase enzymes that contain an oxo-diiron domain (Fe-O-Fe) (7, 8). This domain consists of four transmembrane segments. The loop between the second and third transmembrane segments has a histidine-containing motif ($HX_{3,4}HX_{8-31}HX_{2,3}HH$). Another histidine-rich motif ($HX_{2,3}HH$ or $HX_{2,3}HX_{13-39}HX_{2,3}HH$) follows the fourth transmembrane segment. Sur2p also contains the oxo-diiron motif (9).

The *SUR2* gene was initially identified in a screen for suppressors of *rvs161* mutants (10). Rvs161p is required for endocytosis (11), correct actin localization (12), and viability upon nitrogen, carbon, or sulfur starvation (13). It is similar to amphiphysin, a neuronal protein found in synaptic vesicles that is the autoantigen in stiff-man syndrome (14, 15). The molecular function of Rvs161p and the basis of suppression by mutations in the *SUR* genes have not been identified. However, other *SUR* genes have been found to function in sphingolipid synthesis. *SUR1* is allelic to *CSG1*, a gene required for mannosylation of sphingolipids (5). *SUR4*/*ELO3* encodes a fatty acid elongase required for the synthesis of the very long chain fatty acids (VLCFA)[1] found in ceramide and sphingolipid (16). The genetic relationship between *SUR2*, *SUR1*, and *SUR4* suggests that *SUR2* may also be involved in sphingolipid synthesis.

Based on the homology of Sur2p with Scs7p, required for hydroxylation of the fatty acid of sphingolipids (6), and the genetic relationship between *SUR2* and other sphingolipid synthesis genes, the possibility that *SUR2* is required for hydroxylation of C-4 on the long chain base (LCB) found in sphingolipids was investigated.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The yeast strains used in this study were TDY2037 (*Matα lys2 ura3-52 trp1*Δ *leu2*Δ), TDY2038 (*Matα lys2 ura3-52 trp1*Δ *leu2*Δ *csg2::LEU2*), 2037scs7Δ (*Matα lys2 ura3-52 trp1*Δ *leu2*Δ *scs7::LEU2*), and 6715b (*Matα lys2 ura3-52 trp1*Δ *leu2*Δ *csg2::LEU2 scs7::LEU2*). *SUR2* was disrupted in these strains as described below. Media were prepared, and cells were grown using standard procedures (17). Phytosphingosine, dihydrosphingosine, and sphingosine were purchased from Sigma and added to the growth medium at 25 μM in 1% Tergitol.

Constructing the sur2 Null Mutant—In an unrelated study we isolated a YCp50-based plasmid containing a fragment of yeast DNA that included the amino terminus (through to a *Sau*3A site at codon 120) of the *SUR2* gene. A restriction fragment extending from the *Hin*dIII site 367 base pairs upstream of the start codon of the *SUR2* gene to the *Sa*lI site in YCp50 was subcloned from this plasmid into pUC19. The resulting plasmid was linearized at the *Pst*I site in codon 9 of the *SUR2* gene,

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[1]The abbreviations used are: VLCFA, very long chain fatty acid; LCB, long chain base; FAME, fatty acid methyl ester; HVLCFA, hydroxylated very long chain fatty acid; NVLCFA, unhydroxylated very long chain fatty acid; HVLCFAME, hydroxylated very long chain fatty acid methyl ester; NVLCFAME, unhydroxylated very long chain fatty acid methyl ester; AcOH, acetic acid; MeOH, methanol; BuOH, butanol; $(Et)_2O$, diethyl ether; IPC, inositolphosphorylceramide; MIPC, mannosylinositolphosphorylceramide; $M(IP)_2C$, mannosyldiinositolphosphorylceramide.

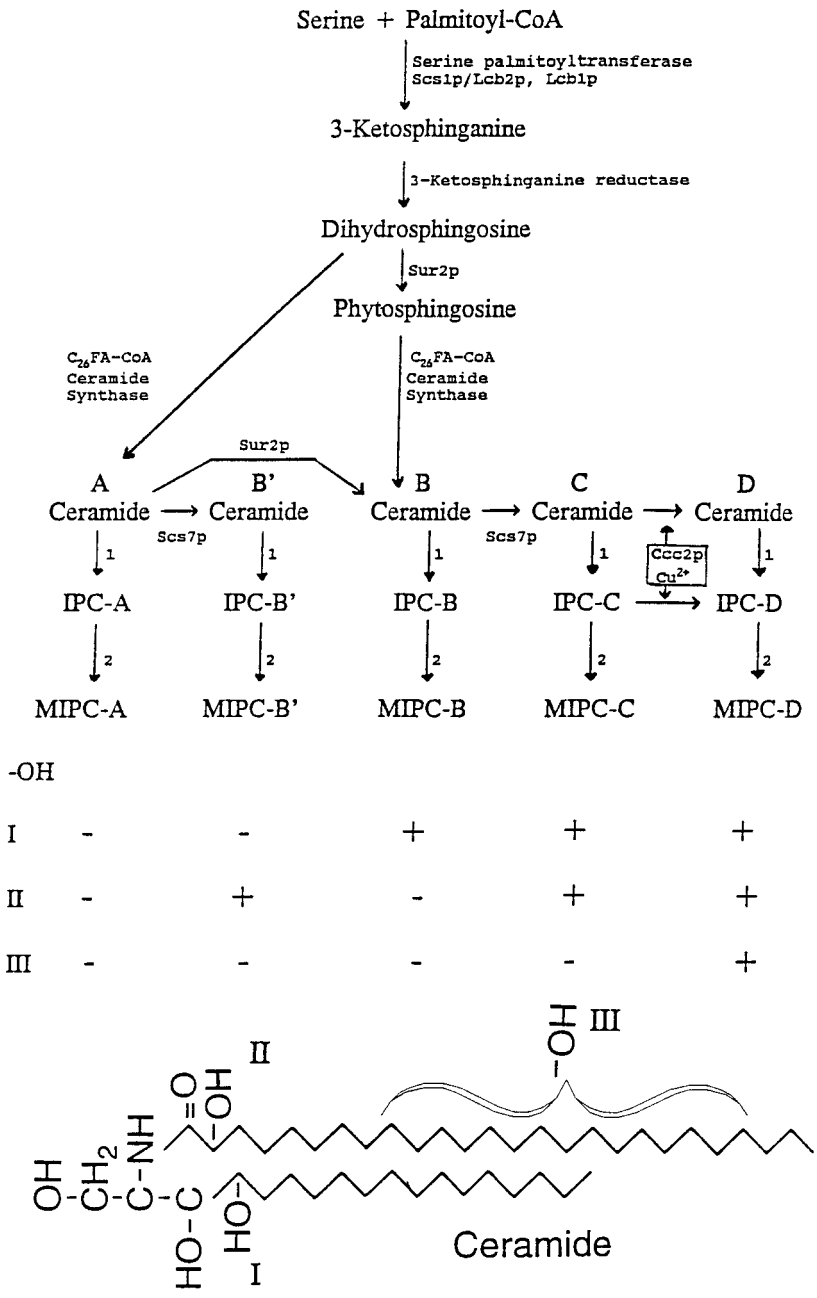


FIG. 1. **The mannosylinositolphosphorylceramide (MIPC) biosynthetic pathway and the structure of ceramide.** Hydroxyl groups on C-1 and C-3 are found on all LCBs. Sites labeled *I* through *III* are potentially hydroxylated. Site I is on C-4 of the LCB, site II is on C-2 of the VLCFA, and site III is also on the VLCFA, but the position has not been determined. Five species (*A*, *B*, *B′*, *C*, and *D*) of ceramides, IPCs, or MIPCs, which differ according to which of the three sites are hydroxylated, are synthesized. In this report, it is shown that hydroxylation of site I and site II requires Sur2p and Scs7p, respectively. Ceramide is converted to IPC by IPC synthase (*reaction 1*), and IPC is converted to MIPC by mannosylation (*reaction 2*).

treated with Bal-31 to remove about 100 base pairs, and incubated with dNTPs, Klenow fragment, ligase, and *Xho*I linkers. A candidate plasmid with a *Xho*I linker at the deletion junction that was missing about 50 base pairs from each side of the original *Pst*I site was used to construct the disrupting plasmid. A *Sal*I fragment carrying the *TRP1* gene was ligated into the *Xho*I site, the *SUR2*-disrupting fragment was cut out of the pUC19 plasmid with *Pvu*II and used in a one-step gene replacement (18). The disruption of *SUR2* was confirmed using a polymerase chain reaction.

Sphingolipid Analysis—Cells were grown in synthetic minimal medium containing 12 nM inositol and 1 μCi/ml [^{3}H]myoinositol for several generations (from A_{600} 0.01 to A_{600} 1.0). Cells (about 5 A_{600} units) were pelleted and washed with 4 mM sodium azide. Lipids were extracted into 600 μl of $CHCl_3$:MeOH (1:1) by vortexing with glass beads, removing the $CHCl_3$:MeOH to a fresh tube and washing the cell pellet and beads with 600 μl of $CHCl_3$:MeOH:H_2O (10:10:3). The pooled extract was dried, alkali-treated, and BuOH-desalted as described previously (5, 19, 20). The samples were analyzed by TLC on silica gel plates using $CHCl_3$:MeOH:AcOH:H_2O (16:6:4:1.6) as the developing solvent (4).

Ceramide Isolation and Analysis—Cells were grown in synthetic medium at 26 °C, spun down, and washed once with H_2O. The cells were vortexed with glass beads in hexane:EtOH (95:5) at 40 A_{600}/ml. The supernatant was transferred to a fresh tube, the pellet and beads were washed with hexane:EtOH, and the pooled extract was dried. The lipids from 60 A_{600} units of cells were alkali-treated by suspending in 1 ml of EtOH:H_2O:Et_2O:pyridine (15:15:5:1) and adding KOH to 0.1 M followed by incubation at 37 °C for 3 h (21). After neutralizing with 1 M AcOH, the sample was dried, BuOH-desalted (20), and dried again. Ceramides were analyzed by TLC on silica gel plates using $CHCl_3$:MeOH:AcOH (95:4.5:0.5) as the developing solvent (22). Plates were sprayed with 10% copper sulfate in 8% orthophosphoric acid and heated for 20 min at 180 °C to char the ceramides (22). The arsenite and borate treated silica gel plates were supplied by Analtech (Newark, DE).

Isolation of Ceramides by Preparative Silica Gel TLC—Ceramides were purified and separated by TLC as described above. The ceramides were visualized by ultraviolet light after spraying the plates with 0.01% 8-anilino-1-napthalenesulfonic acid. The silica gel was scraped off the plate, and the ceramides were eluted by repeated sonication (five times for 10 min each) in 2 ml of $CHCl_3$:MeOH (1:1).

Isolation of Sphingolipids by Preparative Silica Gel TLC—Sphingolipids were extracted from 600 A_{600} units of cells by vortexing with glass beads in 100 ml of $CHCl_3$:MeOH (1:1). The extract was dried, alkali-treated, and BuOH-desalted as described previously (5, 19, 20). The sample was spotted in a line on a silica gel plate and developed using

$CHCl_3$:MeOH:AcOH (95:4.5:0.5). In this system, fatty acids and ceramides migrate while sphingolipids remain at the origin. The material left at the origin was subjected to acid methanolysis for analysis of the fatty acid methyl esters (FAMEs) and LCBs as described below.

Acid Methanolysis of Ceramides and Sphingolipids and Isolation of FAMEs and LCBs—Ceramides and sphingolipids were purified by silica gel TLC as described above. The purified ceramides or sphingolipids were subjected to acid methanolysis by resuspending in 2 ml of HCl:MeOH:H_2O (3:29:4) and incubating at 78 °C for 18 h (23). The FAMEs were recovered by extracting 3 times with 2 ml of hexane (24). The extracts were pooled, dried, and subjected to TLC using petroleum ether:Et_2O (17:3) as the developing solvent (24). Plates were sprayed with 10% copper sulfate in 8% orthophosphoric acid and heated for 20 min at 180 °C to char the FAMEs.

The LCBs were recovered from the hydrolyzed ceramides or sphingolipids by adjusting the pH of the acid hydrolysate (after extraction of FAMEs) to 11.5 using 1 M NaOH and extracting three times with 2 ml of Et_2O (24). The pooled extracts were dried, and the LCBs were separated by silica gel TLC using $CHCl_3$, MeOH, 2.5 M NH_4OH (40:10:1) as developing solvent (24). Plates were sprayed with 0.2% ninhydrin in ethanol and incubated at 100 °C for 5–10 min to visualize the amine-containing LCBs.

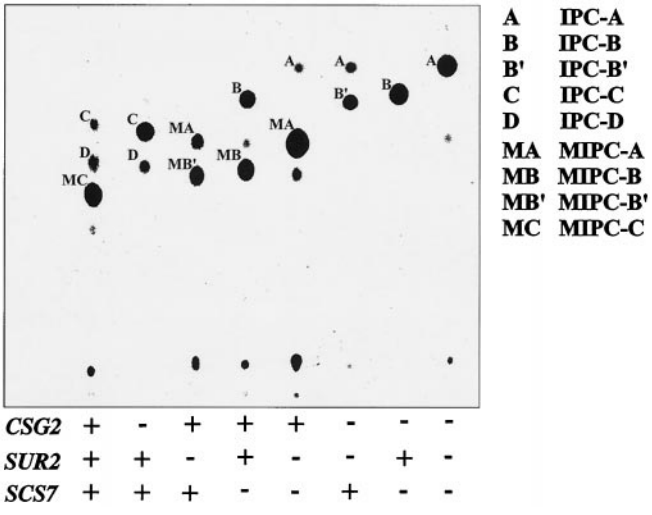


FIG. 2. **Analysis of sphingolipids from wild-type (*lane 1*), *csg2*Δ (*lane 2*), *sur2*Δ (*lane 3*), *scs7*Δ (*lane 4*), *sur2*Δ*scs7*Δ (*lane 5*) *csg2*Δ*sur2*Δ (*lane 6*), *csg2*Δ*scs7*Δ (*lane 7*), and *csg2*Δ*sur2*Δ*scs7*Δ (*lane 8*) cells.** Cells were labeled with [^{3}H]inositol, and sphingolipids were extracted and separated by silica gel TLC as described under "Experimental Procedures." The sphingolipids were visualized by autoradiography. The strains, designated only by the relevant gene disruptions, are derivatives of TDY2037. The hydroxylation states of the sphingolipid species, denoted as *IPC-A*, *IPC-B*, *IPC-B′*, and *IPC-C* or *MIPC-A*, *MIPC-B*, *MIPC-B′*, and *MIPC-C*, are presented in Fig. 1.

RESULTS

*Sphingolipid Synthesis Is Altered in sur2*Δ *Mutant Cells*—The main purpose of this study was to determine if *SUR2* encodes the enzyme that hydroxylates C-4 of the sphingoid moiety of sphingolipids. The *sur2*Δ mutant was constructed as described under "Experimental Procedures." Sphingolipid synthesis in a *sur2*Δ mutant was compared with that of wild-type. Cells were grown for several generations in synthetic minimal medium containing 12 nM inositol with 1 μCi/ml [^{3}H]inositol. [^{3}H]Inositol was incorporated into phosphatidylinositol, inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC) and mannosyldiinositolphosphorylceramide ($M(IP)_2C$). These lipids were extracted out of the cell, alkali-treated to remove phosphatidylinositol, and separated by TLC (Fig. 2). The sphingolipid composition of the *sur2*Δ mutant cells differs from that of wild-type cells (Fig. 2, *lane 1* and *3*). The predominant sphingolipid in wild-type cells is MIPC-C (*lane 1*, *MC*) along with its precursor IPC-C (*C*). IPC-D (*D*) containing dihydroxyl fatty acid is also observed (1). The major sphingolipids in *sur2*Δ mutants (*lane 3*) differ from those of wild-type cells. These sphingolipids are named MIPC-A and MIPC-B′ (*lane 3*, *MA* and *MB′*) for reasons discussed below.

Sphingolipid synthesis in a *sur2*Δ*csg2*Δ double mutant (*lane 6*) was compared with that in a *csg2*Δ mutant (*lane 2*). The *csg2*Δ mutants are defective in mannosylation of inositolphosphorylceramide (17), therefore the sphingolipids in a *csg2*Δ mutant are IPC-C and IPC-D (*lane 2*, *C* and *D*). The *sur2*Δ*csg2*Δ double mutant accumulates two sphingolipid species IPC-A and IPC-B′ (*lane 6*, *A* and *B′*) that are not found in wild-type cells. IPC-A and IPC-B′ are more hydrophobic than is IPC-C (*lane 6*). The two sphingolipids found in a *sur2*Δ single mutant, MIPC-A and MIPC-B′, (*lane 3*, *MA* and *MB′*) are the mannosylated forms of the sphingolipid species seen in a *sur2*Δ*csg2*Δ double mutant, IPC-A and IPC-B′ (*lane 6*, *A* and *B*).

The LCB species present in the inositolphosphorylceramides

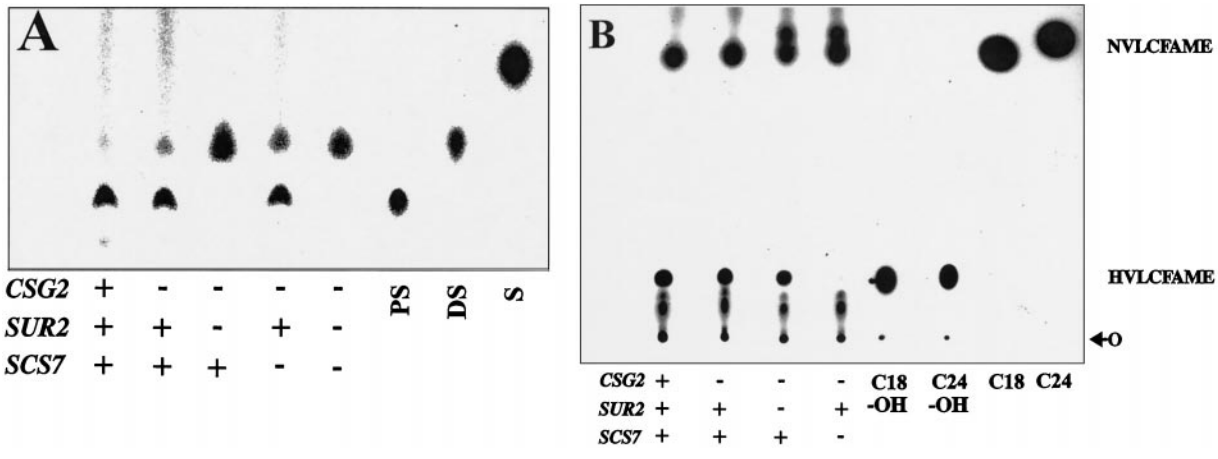


FIG. 3. **Analysis of the LCB moiety (*panel A*) and the VLCFA (*panel B*) of sphingolipids extracted from wild-type (*lane 1*), *csg2*Δ (*lane 2*), *csg2*Δ*sur2*Δ (*lane 3*), *csg2*Δ*scs7*Δ (*lane 4*), and *csg2*Δ*sur2*Δ*scs7*Δ (*lane 5*) mutants.** Sphingolipids were purified by TLC as described under "Experimental Procedures." The sphingolipids were subjected to acid methanolysis, and the liberated FAMEs were extracted with hexane and separated by silica gel TLC (*panel B*). Plates were sprayed with 10% copper sulfate in 8% orthophosphoric acid and heated for 20 min at 180 °C to char the FAMEs. Ten μg of hydroxylated C18 and C24 FAMEs (*lanes 5* and *6*) and nonhydroxylated C18 and C24 FAMEs (*lanes 7* and *8*) standards (Sigma) were spotted. The position of the NVLCFAME and HVLCFAME are labeled in the *right margin*. The spots that migrate just below the NVLCFAMEs as well as those migrating below the HVLCFAMEs are artifacts generated (even in the absence of added lipid) from the acid methanolysis. After extraction of the FAMEs, the pH of the remaining solution was adjusted to 11.5 with NaOH, and the LCBs were extracted with Et_2O and analyzed by TLC (*panel A*). The LCBs were visualized by spraying with 0.2% ninhydrin in ethanol and heating at 100 °C for 5–10 min. Ten μg of phytosphingosine (*PS*), dihydrosphingosine (*DS*), and sphingosine (*S*) standards (Sigma) were spotted.

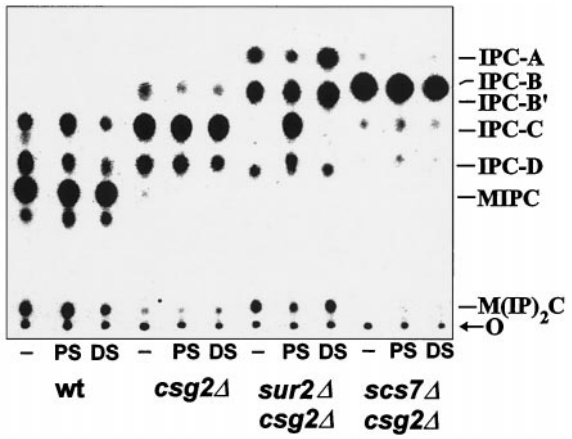


FIG. 4. **Exogenous phytosphingosine restores synthesis of IPC-C to the *csg2*Δ*sur2*Δ double mutant.** Wild-type (*lanes 1–3*), *csg2*Δ (*lanes 4–6*), *csg2*Δ*sur2*Δ (*lanes 7–9*), or *csg2*Δ*scs7*Δ (*lanes 10–12*) mutant cells were grown for several generations in synthetic medium containing 12 nM [^{3}H]inositol at 1 μCi/ml. Where indicated, 25 μM phytosphingosine (*PS*) or dihydrosphingosine (*DS*) was included in the growth medium, which contained 1% Tergitol. Sphingolipids were analyzed as described in Fig. 2.

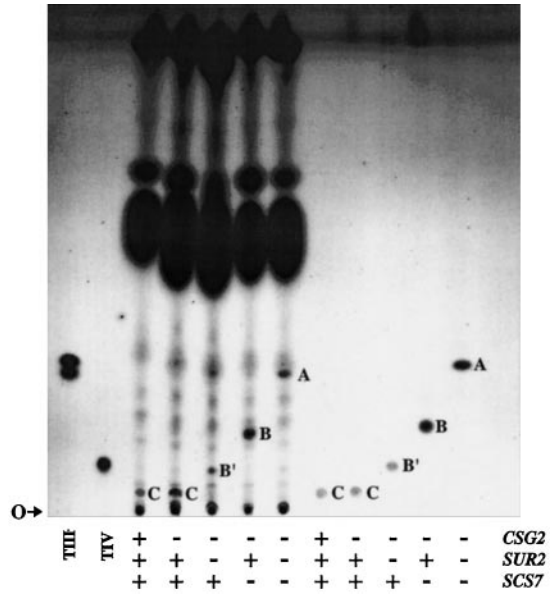


FIG. 5. **Analysis of ceramides from wild-type, *csg2*Δ, *csg2*Δ*sur2*Δ, *csg2*Δ*scs7*Δ, and *csg2*Δ*sur2*Δ*scs7*Δ cells.** Nonpolar lipids were extracted from 10 A_{600} units of wild-type (*lane 3*), *csg2*Δ (*lane 4*), *csg2*Δ*sur2*Δ (*lane 5*), *csg2*Δ*scs7*Δ (*lane 6*), or *csg2*Δ*sur2*Δ*scs7*Δ (*lane 7*) mutant cells, alkali-treated, BuOH-desalted, and separated by TLC as described under "Experimental Procedures." Four μg of bovine type III (sphingosine and unhydroxylated fatty acid, *lane 1*) and type IV (sphingosine and hydroxylated fatty acid, *lane 2*) ceramide standards (Sigma) were spotted. Ceramides from 100 A_{600} units of cells were purified by preparative TLC, and 6% were analyzed (*lanes 8–12*). The hydroxylation states of the ceramide species are shown in Fig. 1.

from *sur2*Δ*csg2*Δ mutants (IPC-A and IPC-B′) was determined. Polar lipids were extracted and alkali-treated to hydrolyze the glycerol-based phospholipids, and the sphingolipids were isolated by preparative TLC as described under "Experimental Procedures." The sphingolipids were subjected to acid methanolysis to hydrolyze both the phosphodiester bond between the inositol and the ceramide, and the amide bond between the sphingoid moiety and the VLCFA. The LCB was extracted and analyzed by TLC (Fig. 3*A*). The LCB in the sphingolipids from the *sur2*Δ mutant cells (*lanes 3* and *5*) is exclusively dihydrosphingosine, while the sphingolipids from cells with a wild-type *SUR2* gene (*lanes 1*, *2*, and *4*) contain primarily phytosphingosine.

The FAMEs released by methanolysis were also analyzed using a TLC system which resolves unhydroxylated very long chain fatty acid methyl esters (NVLCFAME) and hydroxylated very long chain fatty acid methyl esters (HVLCFAME). The sphingolipids from *sur2*Δ*csg2*Δ mutant cells (*lane 3*) contain both hydroxylated and unhydroxylated fatty acids (Fig. 3*B*). Because *sur2*Δ*csg2*Δ mutant cells synthesize two sphingolipids, IPC-A and IPC-B′, these results indicate that IPC-A contains unhydroxylated fatty acids, whereas IPC-B′ contains hydroxylated fatty acids.

The hydroxylation of the VLCFA is dependent on Scs7p (Fig. 3*B*). Cells lacking both Scs7p and Csg2p synthesize an IPC-B species (Fig. 2, *lane 7*) (6, 19), which contains mostly phytosphingosine as the LCB (Fig. 3*A*, *lane 4*) and an unhydroxylated VLCFAME (Fig. 3*B*, *lane 4*). Like IPC-B′, IPC-B can be mannosylated if Csg2p is present (Fig. 2, *lane 4*, *MB*) (6, 19).

In a *sur2*Δ*scs7*Δ*csg2*Δ triple mutant where hydroxylation of C-4 of the LCB is blocked by deletion of *SUR2*, hydroxylation of the VLCFA is blocked by deletion of *SCS7*, and mannosylation is blocked by deletion of *CSG2*, the only sphingolipid synthesized is the very hydrophobic IPC-A (Fig. 2, *lane 8*, *A*). The IPC-A species can be mannosylated if Csg2p is present (Fig. 2, *lane 5*, *MA*). The *sur2*Δ mutants accumulate some IPC-A or MIPC-A even when Scs7p is present (Fig. 2, *lanes 3* and *6*, *MA* and *A*) suggesting that phytosphingosine-containing substrates are preferred by Scs7p over dihydrosphingosine-containing substrates. These data (summarized in the model shown in Fig. 1) support the proposal that Sur2p is the hydroxylase that converts dihydroceramide to phytoceramide.

The Sphingolipid Synthesis Defect Conferred by Deletion of sur2 Is Corrected by Exogenous Phytosphingosine but Not Dihydrosphingosine—S. cerevisiae cells can incorporate exogenous phytosphingosine into sphingolipids (25). As would be predicted if Sur2p is required for hydroxylation of C-4 on the LCB, exogenous phytosphingosine, but not dihydrosphingosine, restores synthesis of IPC-C to a *sur2*Δ*csg2*Δ mutant (Fig. 4, *lanes 8* and *9*). A *sur2*Δ*scs7*Δ*csg2*Δ triple mutant, that normally makes IPC-A, makes IPC-B in the presence of phytosphingosine (data not shown). These observations provide further evidence that the altered sphingolipids that accumulate in the *sur2*Δ*csg2*Δ and *sur2*Δ*csg2*Δ*scs7*Δ mutants (IPC-B′ and IPC-A, respectively) differ from the sphingolipids in *csg2*Δ and *csg2*Δ*scs7*Δ mutants (IPC-C and IPC-B, respectively) in the LCB. Addition of phytosphingosine to the *scs7*Δ*csg2*Δ mutant does not result in any IPC-C synthesis (Fig. 4, *lane 11*), because the IPC-B that accumulates in the *scs7*Δ mutant arises from failure to hydroxylate the VLCFA (6).

Deleting the SUR2 or SCS7 Gene Reduces Hydroxylation of Ceramides—The IPCs are synthesized by the transfer of phosphoinositol from phosphatidylinositol to ceramide. Comparison of ceramides isolated from the *sur2*Δ*csg2*Δ, *scs7*Δ*csg2*Δ and the *sur2*Δ*scs7*Δ*csg2*Δ mutants with ceramides from wild-type or *csg2*Δ mutant cells demonstrates that the altered mobility of the sphingolipids arises from differences in the ceramide moiety of the sphingolipids. Yeast ceramides were analyzed by TLC (Fig. 5). The predominant ceramide in wild-type and in *csg2*Δ mutant cells (*lanes 3* and *4*), labeled "*C*" (for C-ceramide) because it is the ceramide of IPC-C, migrates slower in this TLC system than the bovine hydroxylated ceramide standard (Sigma type IV, *lane 2*) which consists of sphingosine and a hydroxylated fatty acid. Analysis of the LCB and VLCFA of the C-ceramide by TLC following methanolysis confirms that it contains phytosphingosine (Fig. 6*A*, *lanes 5* and *6*) and a HVLCFA (Fig. 6*B*, *lanes 6* and *7*) (1, 26). The slower mobility of yeast ceramide compared with hydroxylated bovine ceramides

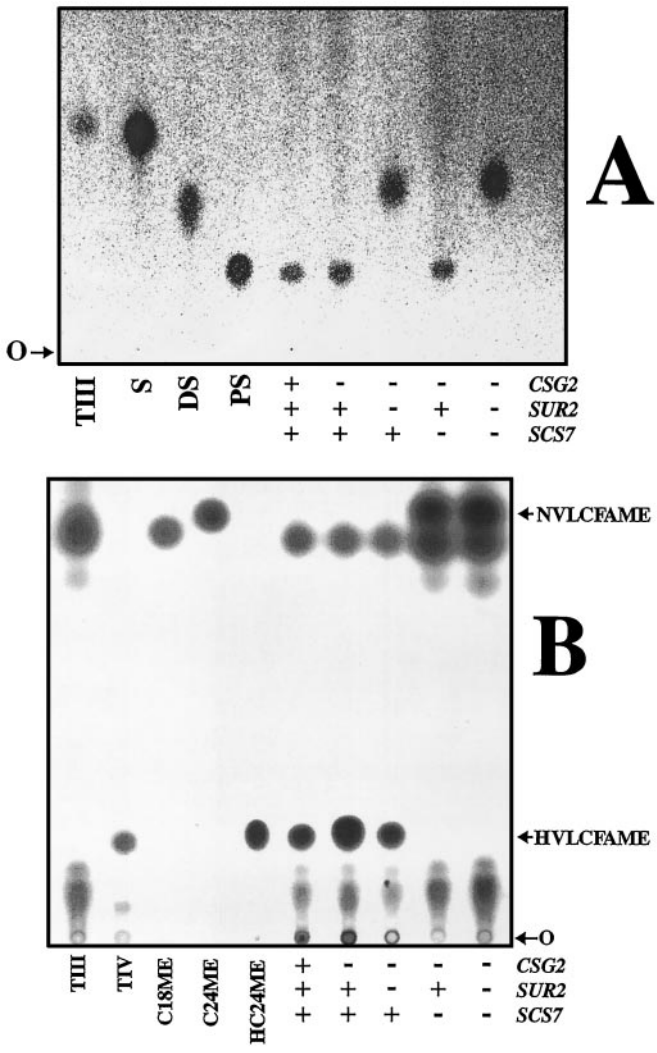


FIG. 6. **The LCBs and the FAMEs from A-, B′-, B-, and C-ceramides were analyzed by silica gel TLC.** Ceramides purified from 100 A_{600} units of cells (see Fig. 5) were subjected to acid methanolysis, and FAMEs and LCBs were extracted for analysis as described in Fig. 3. *A*, the LCBs were separated by silica gel TLC. Standards were the LCB derived from acid methanolysis of 4 μg of Sigma type III bovine ceramide (*lane 1*) and 10 μg of sphingosine (*S*), dihydrosphingosine (*DS*), and phytosphingosine (*PS*). The LCBs were visualized by spraying with 0.2% ninhydrin in ethanol and heating at 100 °C for 5–10 min. *B*, the FAMEs were also separated by TLC. Standards were the FAMEs derived from acid methanolysis of Sigma type III and type IV bovine ceramides (4 μg) (*lanes 1* and *2*), and 10 μg of C18 FAME, C24 FAME, and hydroxylated C24 FAME (*lanes 3–5*). The plate was sprayed with 10% copper sulfate in 8% orthophosphoric acid and heated for 20 min at 180 °C to char the FAMEs.

is expected, since it contains an additional hydroxyl group (phytosphingosine *versus* sphingosine).

The major ceramide from *sur2*Δ mutant cells (B′-ceramide) has a mobility similar to the hydroxylated bovine ceramide standard. Ceramides having dihydrosphingosine might be expected to have similar hydrophobicity to those having sphingosine. The LCB from the B′-ceramide is dihydrosphingosine (Fig. 6*A*, *lane 7*) and the VLCFA is hydroxylated (Fig. 6*B*, *lane 8*).

The B-ceramide that accumulates in the *scs7*Δ mutant cells contains phytosphingosine (Fig. 6*A*, *lane 8*) and unhydroxylated VLCFA (Fig. 6*B*, *lane 9*). The mobility of the ceramide in *scs7*Δ mutant cells is quite distinct from that in *sur2*Δ mutant cells (Fig. 5, *lanes 5* and *6*). Either hydroxylation of the VLCFA increases the hydrophilicity of the ceramide less than does the hydroxylation of the LCB, or these species interact differently with the silica gel matrix.

The A-ceramide that is present in the *sur2*Δ*scs7*Δ double mutant (Fig. 5, *lane 7*) migrates with the unhydroxylated bovine ceramide standards as would be expected if it lacks hydroxyl groups on both C4 of the LCB and on the VLCFA (Fig. 5, *lanes 1* and *7*). The LCB of the A-ceramide is dihydrosphingosine (Fig. 6*A*, *lane 9*) and the VLCFA is unhydroxylated (Fig. 6*B*, *lane 10*). The absence of vicinal hydroxyl groups (C-3 and C-4 of phytosphingosine) on ceramide from *sur2*Δ mutants is also indicated by the effect of the glycol-complexing ions arsenite and borate on the chromatographic behavior of the B′- and A-ceramides (Fig. 7). The complex between vicinal hydroxyl groups with arsenite increases their mobility on silica gel, while the borate complex decreases their mobility (27, 28). The mobility of C-ceramide (from wild-type and *csg2*Δ mutant cells) and B-ceramide (from *csg2*Δ*scs7*Δ mutant cells) is greatly increased by addition of $NaAsO_2$ to the silica gel (compare Fig. 7, *B* to *A*, *lanes 1*, *2*, and *4*) and reduced by addition of $Na_2B_4O_7$ (Fig. 7*C*, *lanes 1*, *2*, and *4*), indicating that these ceramides contain the C-3,4 vicinal hydroxyl groups of phytoceramide. The mobility of the B′-ceramide (from *csg2*Δ*sur2*Δ mutant cells) and A-ceramide (from *csg2*Δ*sur2*Δ*scs7*Δ mutant cells) is much less affected by arsenite or borate, consistent with the conclusion that they have dihydrosphingosine instead of phytosphingosine as the LCB.

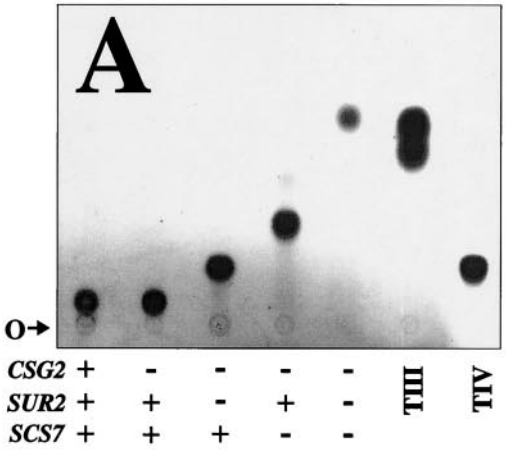
Deletion of the SUR2 Gene Suppresses Ca^{2+} Sensitivity of csg2 Mutants—Cells lacking the *CSG2* gene are defective in mannosylation of inositolphosphorylceramides and therefore accumulate the inositolphosphorylceramide, IPC-C (Fig. 2, *lane 2*) (5, 19). Overaccumulation of IPC-C or a related metabolite confers Ca^{2+}-sensitivity. Mutations in a variety of genes required for the synthesis of IPC-C suppress the Ca^{2+}-sensitive phenotype of the *csg2* mutants. For example, deletion of *SCS7*, which encodes the enzyme that hydroxylates the VLCFA suppresses the Ca^{2+} sensitivity of the *csg2* mutant (Fig. 8) (6, 19). Therefore, the effect of deletion of *SUR2* on the Ca^{2+} sensitivity of the *csg2*Δ mutant was investigated. As shown in Fig. 8, deletion of the *SUR2* gene reverses the Ca^{2+} sensitivity of a *csg2* mutant.

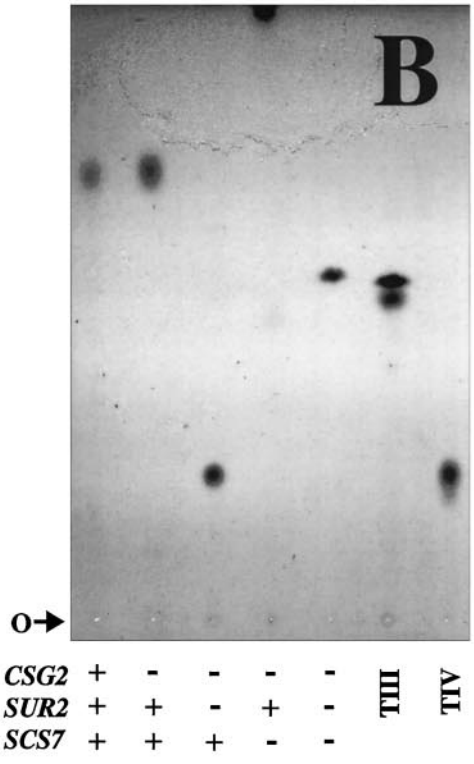
DISCUSSION

SUR2 Is Required for the Hydroxylation of C-4 of the LCB and SCS7 Is Required for Hydroxylation of the VLCFA of Ceramides—The effect of deleting *SUR2* on the hydroxylation of C-4 on the LCB of ceramide and sphingolipid and the sequence similarity between Sur2p and a family of desaturases/hydroxylases indicate that Sur2p catalyzes the hydroxylation of C-4. The LCB of ceramides and sphingolipids in *sur2*Δ mutants is dihydrosphingosine instead of the phytosphingosine predominantly found in wild-type cells. Exogenous phytosphingosine restores synthesis of sphingolipids with a phytosphingosine LCB in *sur2*Δ mutants.

The substrate (dihydrosphingosine or dihydroceramide) for Sur2p has not been identified. Since hydroxylation of C-4 is not required for ceramide or sphingolipid synthesis, either dihydrosphingosine or phytosphingosine can serve as substrate for ceramide synthase, and either dihydroceramide or phytoceramide can serve as substrate for IPC synthase. *S. cerevisiae* cells contain both dihydrosphingosine and phytosphingosine, and inhibition of ceramide synthase by fumonisin B_1 causes the accumulation of both LCBs (29). However, it is not known whether phytosphingosine comes from *de novo* synthesis or from turnover of ceramide and sphingolipid.

Scs7p is also a member of this family of hydroxylases/desaturases, but it is responsible for hydroxylation of the VLCFA rather than the LCB. Failure to hydroxylate C4 of the LCB





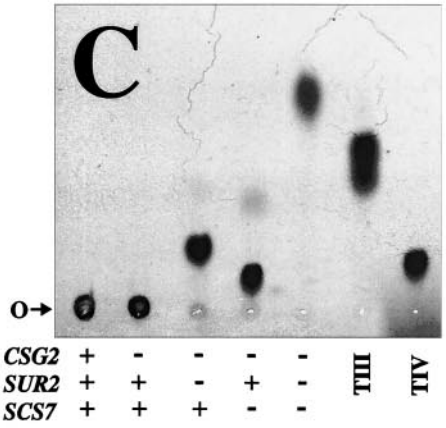


FIG. 7. **Effect of arsenite and borate on the relative chromatographic mobilities of the C (*lanes 1* and *2*)-, B′ (*lane 3*)-, B (*lane 4*)-, and A (*lane 5*)-ceramides.** The isolated ceramides used in the experiment described in Fig. 6 were analyzed by TLC on silica gel plates without (*panel A*) or with either 1% sodium *meta* arsenite (*panel B*) or 1% sodium borate (*panel C*) as described by Karlsson and Pascher (27). The borate plate and the untreated plate were run once in $CHCl_3$:CH_3OH (95:5), while the arsenite plate was run twice in $CHCl_3$:CH_3OH:AcOH (95:4.5:0.5).

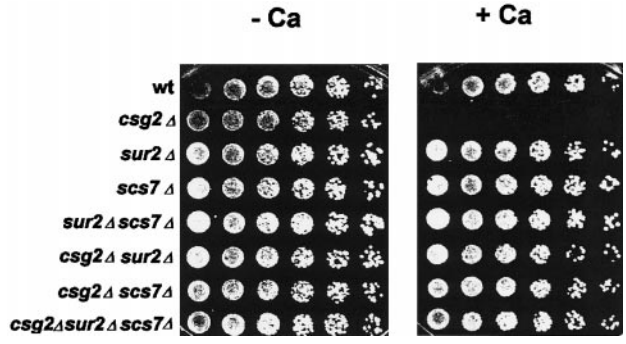


FIG. 8. **Suppression of the Ca^{2+}-sensitive phenotype of *csg2*Δ mutants by deletion of *SUR2* or *SCS7*.** Cells were grown in synthetic medium to an OD_{600} of 0.1 and serially diluted (left to right, 1:5) into the wells of a microtiter plate. Cells were transferred by metal prong to SD agar plates without (−*Ca*) or with an additional 50 mM $CaCl_2$ (+*Ca*). The plates were incubated at 26 °C for three days.

decreases the Scs7p-catalyzed hydroxylation of the VLCFA, indicating hydroxylation occurs subsequent to ceramide formation. Furthermore, the ceramides from *SCS7*$^+$ cells are hydroxylated, while those from *scs7*Δ mutant cells are not suggesting that the substrate for Scs7p is ceramide. However, it is not yet known whether most of the free ceramides in the cell arise from *de novo* synthesis or from turnover of sphingolipid, so it remains to be determined whether the substrate for Scs7p is free ceramide or inositolphosphorylceramide.

Martin and co-workers (16) recently reported that cells lacking the elongase encoded by *ELO3*/*SUR4* accumulate relatively high levels of hydroxylated C16 fatty acids. We have found that *elo3* mutant cells incorporate fatty acids with shorter than normal chain lengths into ceramide.[2] Therefore, it will be interesting to determine whether the hydroxylated C16 fatty acids in the mutants arise from Scs7p-catalyzed hydroxylation of the (shorter than normal) fatty acids on the ceramide.

Sur2p and Scs7p Are Members of a Family of Cytochrome b_5-dependent Enzymes Located in the Endoplasmic Reticulum—Ceramide and IPC-C are synthesized in the endoplasmic reticulum (4) which appears to be the location of Scs7p and Sur2p as well. Both Scs7p and Sur2p contain C-terminal sequences (KMKYE and VKKEK), matching a consensus sequence specifying retention in the endoplasmic reticulum (6, 30). In *S. cerevisiae*, all five proteins that are members of the oxo-diiron family appear to reside in the endoplasmic reticulum. Along with Sur2p and Scs7p, these are δ-9 fatty acid desaturase (Ole1p), C-4 sterol methyl oxidase (Erg25p), and C-5 sterol desaturase (Erg3p). The oxo-diiron centers in these enzymes are believed to receive electrons from either cytochrome b_5 or a cytochrome b_5-like domain. Scs7p and Ole1p contain cytochrome b_5-like domains at their N and C termini respectively (6, 31). Cytochrome b_5 may function to transfer electrons to Sur2p and the other two enzymes. Cytochrome b_5 reductase may catalyze the reduction of both cytochrome b_5 and the cytochrome b_5-like domains on Scs7p and Ole1p.

*Suppressors of the Ca^{2+}-sensitive Phenotype of csg2*Δ *Mutants, as Well as Suppressors of the Pleiotropic Phenotypes of rvs161 Mutants, Identify Sphingolipid Synthesis Genes*—The Ca^{2+} sensitivity of *csg2* mutants is suppressed by deletion of

[2] D. Haak, K. Gable, T. Beeler, and T. Dunn, unpublished observations.

SUR2. Other mutations in sphingolipid biosynthetic genes (subunits of serine palmitoyltransferase, *LCB1*, *SCS1*/*LCB2*; ceramide hydroxylase, *SCS7*; fatty acid elongases, *ELO2*/*SUR5*/*FEN1*, *ELO3*/*SUR4*; and fatty acid synthetase, *FAS2*) also suppress the Ca^{2+} sensitivity of *csg* mutants. These mutations either decrease the rate of sphingolipid synthesis or alter the sphingolipids that are synthesized. The *CSG2* and *CSG1* genes are required for mannosylation of IPC to form MIPC. In the absence of mannosylation, IPC-C overaccumulation is observed. It appears that decreasing the accumulation of IPC-C or a related metabolite or altering its structure (to IPC-B, IPC-B′, or IPC-A?) reverses the Ca^{2+} sensitivity. It is hoped that continued analysis of suppressor mutants will identify more genes that function in sphingolipid synthesis and identify the Ca^{2+} target that triggers cell death.

The genetic relationship between suppressors of the *csg2*Δ mutant and suppressors of the *rvs161* mutant suggest a role for sphingolipid in some Rvs161p-dependent process. Three genes (*SUR1*, *SUR2*, and *SUR4*) (12) that mutate to suppress *rvs161* mutants (10) are related to *CSG1* and *CSG2* or to genes that mutate to suppress the Ca^{2+}-sensitive phenotype of *csg1*Δ and *csg2*Δ mutants. The *sur1*, *sur2*, and *sur4* mutants have altered phospholipid compositions and abnormal morphologies in stationary phase (10). *SUR1*, which is allelic to *CSG1* (5), is a high copy suppressor of *csg2*Δ mutants (5, 32). Both *SUR1*/*CSG1* and *CSG2* are required for mannosylation of IPC (5, 19). *SUR2* encodes the enzyme that hydroxylates C-4 of dihydroceramide. *SUR4*/*ELO3* and *SUR5*/*ELO2*/*FEN1* encode fatty acid elongases required for the synthesis of the C26 fatty acids found in ceramide and sphingolipids (16).

S. cerevisiae Cells Do Not Require Sur2p- or Scs7p-mediated Hydroxylation for Growth or Synthesis of Mature Sphingolipid—The physiological function of Sur2p- and Scs7p-mediated hydroxylation is not known. Growth of *S. cerevisiae* cells does not depend on hydroxylation of either the C-4 of the LCB or the VLCFA moieties of ceramides and sphingolipids. Cells lacking serine palmitoyltransferase can utilize exogenous dihydrosphingosine or phytosphingosine but not sphingosine, the main long chain base in mammals (25). Since *sur2*Δ mutants do not synthesize phytosphingosine, it is not the lack of a C-4 hydroxyl group that precludes sphingosine from substituting as the LCB in ceramide synthesis.

Deletion of *SUR2* greatly increases the resistance of cells to the *Pseudomonas syringae* cyclic lipodepsipeptide syringomycin (33) and to the morpholine fungicide fenpropimorph,[2] an inhibitor of several enzymes in the ergosterol synthesis pathway. As discussed above, deletion of *SUR2* also suppresses the Ca^{2+}-sensitive phenotype of *csg2*Δ mutants and the pleiotropic effects of *rvs161* mutations. Elucidation of the mechanism by which blocking hydroxylation of the LCB C-4 increases the ability of cells to tolerate syringomycin, fenpropimorph, and high Ca^{2+} concentrations after *CSG2* deletion, and *RVS161* deletion may provide clues as to how C-4 hydroxylation affects the functional properties of the LCB, ceramide, and sphingolipids. In addition, the *sur2*Δ mutant can be used to identify genetically related genes that encode proteins whose functional properties are effected by C-4 hydroxylation.

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