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Haak, Dale; Gable, Ken; Beeler, Troy; and Dunn, Teresa, "Hydroxylation of *Saccharomyces cerevisiae* Ceramides Requires Sur2p and Scs7p" (1997). *Uniformed Services University of the Health Sciences*. 52. [https://digitalcommons.unl.edu/usuhs/52](https://digitalcommons.unl.edu/usuhs/52)

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Hydroxylation of Saccharomyces cerevisiae Ceramides Requires Sur2p and Scs7p*

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The Saccharomyces cerevisiae SC57 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 SC57 nor SUR2 are essential for growth, and lack of the Scs7p- or Sur2p-dependent hydroxylation does not prevent the synthesis of mannosylinositolphosphorylceramide, the mature sphingolipid found in yeast. Deletion of either gene suppresses the Ca2+-sensitive phenotype of csg2Δ 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 mammalian cells, sphingoid moieties are 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 Cu2+ 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 (HX3,4HX8,9HX13,14HX20,21HH). Another histidine-rich motif (HX5,6HH or HX23,24HX29,30HX31,32HH) 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 rsa1Δ mutants (10). Rsa1p 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 Rsa1p and the basis of suppression by mutations in the SUR2 gene 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 YD6037 (Mata lys2 ura3-52 trp1Δ leu2Δ), YD6038 (Mata lys2 ura3-52 trp1Δ leu2Δ csg2::LEU2), 2057esc7A (Mata lys2 ura3-52 trp1Δ leu2Δ csg2::LEU2), and 6715b (Mata lys2 ura3-52 trp1Δ leu2Δ csg2::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 SacI site at codon 120) of the SUR2 gene. A restriction fragment extending from the SacI site to the SUR2 gene, was cloned into the HindIII site of pRS316 and expressed from the ADH1 promoter. The resulting plasmid was linearized at the PstI site in codon 9 of the SUR2 gene.

*This work was supported by National Institutes of Health Grant GM 51891 and Uniformed Services University of the Health Sciences Grants CO71Cw and 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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1The 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; HVLCFAME, hydroxylated very long chain fatty acid methyl ester; AcOH, acetic acid; MeOH, methanol; BuOH, butanol; (Et)2O, diethyl ether; IPC, inositolphosphorylceramide; MIPC, mannosylinositolphosphorylceramide; M(IP)3C, mannosylinositolphosphorylceramide.
treated with Bal-31 to remove about 100 base pairs, and incubated with DNA, Klenow fragment, ligase, and XhoI linkers. A candidate plasmid with an XhoI linker at the deletion junction that was missing about 50 base pairs from each side of the original PstI site was used to construct the disrupting plasmid. A SalI fragment carrying the TRP1 gene was ligated into the XhoI site. The disruption of SUR2 was confirmed using a polymerase chain reaction.

**Sphingolipid Analysis**—Cells were grown in synthetic minimal medium containing 12 mM inositol and 1 mCi/ml [3H]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 ml of CHCl₃:MeOH (1:1) by vortexing with glass beads. 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 CHCl₃:MeOH:AcOH:H₂O (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 H₂O. 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:H₂O:Et₂O: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 CHCl₃: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 CHCl₃: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 CHCl₃:MeOH (1:1). 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:H₂O:Et₂O: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 CHCl₃: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-naphthalenesulfonic 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 CHCl₃:MeOH (1:1). 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:H₂O:Et₂O: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 CHCl₃: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).

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Cells were labeled with $^3$H]inositol, and sphingolipids and LCBs 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$_2$O (3:29:4) and incubating at 78 °C for 18 h (23). The FAMEs were subjected to acid methanolysis by resuspending in 2 ml of HCl:MeOH:H$_2$O (3:29:4) and incubating at 78 °C for 18 h (23).

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 sur2Δ mutant was constructed as described under "Experimental Procedures." Sphingolipid synthesis in a sur2Δ 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 mannosylidinositolphosphorylceramide (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 sur2Δ 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 sur2Δ 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 sur2Δcsg2Δ double mutant (lane 6) was compared with that in a csg2Δ mutant (lane 2). The csg2Δ mutants are defective in mannosylation of inositolphosphorylceramide (17), therefore the sphingolipids in a csg2Δ mutant are IPC-C and IPC-D (lane 2, C and D). The sur2Δcsg2Δ 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 sur2Δ single mutant, MIPC-A and MIPC-B' (lane 3, MA and MB') are the mannosylated forms of the sphingolipid species seen in a sur2Δcsg2Δ double mutant, IPC-A and IPC-B' (lane 6, A and B).

The LCB species present in the inositolphosphorylceramides...
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The Sphingolipid Synthesis Defect Conferred by Deletion of SUR2 Is Corrected by Exogenous Phytosphingosine but Not Dihydrophosphinosine—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 dihydrophosphinosine, restores synthesis of IPC-C to a sur2Δcsg2Δ mutant (Fig. 4, lanes 8 and 9). A sur2Δcs7Δcsg2Δ 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 sur2Δcsg2Δ and sur2Δcsg2Δcs7Δ mutants (IPC-B’ and IPC-A, respectively) differ from the sphingolipids in csg2Δ and csg2Δcs7Δ mutants (IPC-C and IPC-B, respectively) in the LCB. Addition of phytosphingosine to the cs7Δcsg2Δ mutant does not result in any IPC-C synthesis (Fig. 4, lane 11), because the IPC-B that accumulates in the cs7Δ mutant arises from failure to hydroxylate the VLCFA (6).

Deletion of the SUR2 or CS7 Gene Reduces Hydroxylation of Ceramides—The IPCs are synthesized by the transfer of phosphinositol from phosphatidylinositol to ceramide. Comparison of ceramides isolated from the sur2Δcsg2Δ, cs7Δcsg2Δ and sur2Δcsg2Δcs7Δ mutants with ceramides from wild-type or csg2Δ 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 csg2Δ mutant cells (lanes 3 and 5), 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. 6A, lanes 5 and 6) and a HV-LCFA (Fig. 6B, lanes 6 and 7) (1, 26). The slower mobility of yeast ceramide compared with hydroxylated bovine ceramides from sur2Δcsg2Δ 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 phospodiester 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. 3A). The LCB in the sphingolipids from the sur2Δ mutant cells (lanes 3 and 5) is exclusively dihydrophosphinosine, 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 sur2Δcsg2Δ mutant cells (lane 3) contain both hydroxylated and unhydroxylated fatty acids (Fig. 3B). Because sur2Δcsg2Δ 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. 3B). 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. 3A, lane 4) and an unhydroxylated VLCFA (Fig. 3B, lane 4). Like IPC-B’, IPC-B can be mannosylated if Csg2p is present (Fig. 2, lane 4, MB) (6, 19).

In a sur2Δcs7Δcsg2Δ 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 CS7, and mannoxylation 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 sur2Δ 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 dihydrophosphinosine-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 FAMEs released by methanolysis were also analyzed by TLC following methanolysis. Four nonpolar lipids were extracted from 10 Aoa cells of wild-type (lane 3), csg2Δ (lane 4), sur2Δ (lane 5), csg2Δsur2Δ (lane 6), or csg2Δsur2Δcs7Δ (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 Aoa 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.

The hydroxylation of the VLCFA is dependent on Scs7p (Fig. 3B). 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. 3A, lane 4) and an unhydroxylated VLCFA (Fig. 3B, lane 4). Like IPC-B’, IPC-B can be mannosylated if Csg2p is present (Fig. 2, lane 4, MB) (6, 19).

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The FAMEs released by methanolysis were also analyzed by TLC following methanolysis. Four nonpolar lipids were extracted from 10 Aoa cells of wild-type (lane 3), csg2Δ (lane 4), sur2Δ (lane 5), csg2Δsur2Δ (lane 6), or csg2Δsur2Δcs7Δ (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 Aoa 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.
Ceramide Hydroxylation by Scs7p and Sur2p

**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_{rev} 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 methanalysis 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 methanalysis 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.

The major ceramide from sur2Δ 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. 6A, lane 7) and the VLCFA is hydroxylated (Fig. 6B, lane 8).

The B-ceramide that accumulates in the scs7Δ mutant cells contains phytosphingosine (Fig. 6A, lane 8) and unhydroxylated VLCFA (Fig. 6B, lane 9). The mobility of the ceramide in scs7Δ mutant cells is quite distinct from that in sur2Δ 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 sur2Δscs7Δ 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. 6A, lane 9) and the VLCFA is unhydroxylated (Fig. 6B, lane 10). The absence of vicinal hydroxyl groups (C-3 and C-4 of phytosphingosine) on ceramide from sur2Δ 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 csg2Δ mutant cells) and B-ceramide (from csg2Δscs7Δ mutant cells) is greatly increased by addition of NaAsO2 to the silica gel (compare Fig. 7, A to B, lanes 1, 2, and 4) and reduced by addition of NaB\(_4\)O\(_7\) (Fig. 7C, 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 csg2Δsur2Δ mutant cells) and A-ceramide (from csg2Δsur2Δscs7Δ 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 are required for the synthesis of IPC-C or a related metabolite, conferring 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 csg2Δ 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 sur2Δ mutants is dihydrosphingosine instead of the phytosphingosine predominantly found in wild-type cells. Exogenous phytosphingosine restores synthesis of sphingolipids with a phytosphingosine LCB in sur2Δ 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 B1 causes the inhibition of ceramide synthase by fumonisin B1 causes the phenotype of the csg2 mutant. For example, deletion of CSG2, 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 csg2Δ mutant was investigated. As shown in Fig. 8, deletion of the SUR2 gene reverses the Ca\(^{2+}\)-sensitivity of a csg2 mutant.
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 scs7Δ 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 elo3Δ mutant cells incorporate fatty acids with shorter than normal chain lengths into ceramide. 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 b5-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 b5 or a cytochrome b5-like domain. Scs7p and Ole1p contain cytochrome b5-like domains at their N and C termini respectively (6, 31). Cytochrome b5 may function to transfer electrons to Sur2p and the other two enzymes. Cytochrome b5 reductase may catalyze the reduction of both cytochrome b5 and the cytochrome b5-like domains on Scs7p and Ole1p.

Suppressors of the Ca2+-sensitive Phenotype of csg2Δ Mutants, as Well as Suppressors of the Pleiotropic Phenotypes of rvs161 Mutants, Identify Sphingolipid Synthesis Genes—The Ca2+-sensitivity of csg2 mutants is suppressed by deletion of

1% sodium borate (panel C) as described by Karlsson and Pascher (27). The borate plate and the untreated plate were run once in CHCl3:CH3OH:AcOH (95:5:0.5), while the arsenite plate was run twice in CHCl3:CH3OH:AcOH (95:4.5:0.5).
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 overaccumulates, 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\(\Delta\) mutant can be used to identify genetically related genes that encode proteins whose functional properties are effected by C-4 hydroxylation.

**Acknowledgments**—We thank Ann Moser for the protocol for visualizing ceramides using 8-anilino-1-naphthalene sulfonic acid, and Alan Akers (BASF) for the gift of fenpropimorph.

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