Syringomycin Action Gene SYR2 Is Essential for Sphingolipid 4-Hydroxylation in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* gene SYR2, necessary for growth inhibition by the cyclic lipodepsipeptide syringomycin E, is shown to be required for 4-hydroxylation of long chain bases in sphingolipid biosynthesis. Four lines of support for this conclusion are presented: (a) the predicted Syr2p shows sequence similarity to diiron-binding membrane enzymes involved in oxygen-dependent modifications of hydrocarbon substrates, (b) yeast strains carrying a disrupted SYR2 allele produced sphingolipid long chain bases lacking the 4-hydroxyl group present in wild type strains, (c) 4-hydroxylase activity was increased in microsomes prepared from a SYR2 overexpression strain, and (d) the syringomycin E resistance phenotype of a syr2 mutant strain was suppressed when grown under conditions in which exogenous 4-hydroxysphingoid long chain bases were incorporated into sphingolipids. The syr2 strain produced wild type levels of sphingolipids, substantial levels of hydroxylated very long chain fatty acids, and the full complement of normal yeast sphingolipid head groups. These results show that the SYR2 gene is required for the 4-hydroxylation reaction of sphingolipid long chain bases, that this hydroxylation is not essential for growth, and that the 4-hydroxy group of sphingolipids is necessary for syringomycin E action on yeast.

Syringomycin E is a member of a family of cyclic lipodepsipeptides produced by strains of the plant bacterium *Pseudomonas syringae* pv. *syringae* (1). Traditionally regarded as a virulence factor in a variety of bacterial necrotic diseases of plants (2), syringomycin E and its analogs also possess anti-fungal properties, and it has been suggested that these metabolites are fungal antagonists that aid survival of the producing bacteria on plants (3, 4).

How these compounds produce their toxic effects is unknown, but past physiological studies have shown that syringomycin E targets primarily the plasma membrane (1, 5, 6). To further investigate the molecular mechanisms of action of this bioactive compound, resistant mutants of *Saccharomyces cerevisiae* were isolated to identify genes that encode proteins necessary for growth inhibition by syringomycin E (7).

Several of the mutants were deficient in sterols, and one group was complemented by the gene SYR1 (identical to ERG3), which encodes sterol C-5,6 desaturase of the ergosterol biosynthetic pathway (8). These findings, when combined with results from binding (9) and lipid bilayer (10) studies, indicate that sterols influence the interaction of syringomycin E with the target plasma membrane.

Syringomycin E action in yeast was more recently shown to require a second, nonsterol biosynthetic gene, SYR2 (11). SYR2 is identical to SYR2, which was identified in a screen for mutants that suppress the impaired recovery of *rsv161* strains from nutritional starvation (12). Syringomycin E-resistant syr2 mutants showed altered glycerophospholipid levels, and the SYR2 gene product was localized to the endoplasmic reticulum (11). Nevertheless, the precise function of Syr2p was unclear from these studies.

In addition to sterols and glycerophospholipids, sphingolipids are major lipid components of the plasma membrane (13). Ubiquitous in eukaryotic cells, sphingolipids all possess a sphingoid long chain base with mainly, in fungi and plants, a hydroxyl group at the C-4 position (phytosphingosine) or, in animals, a double bond at the C-4,5 position (sphingosine). Sphingolipids serve numerous roles, including mediating cell-cell interactions, anchoring membrane proteins, acting as enzyme co-factors (14), and serving as receptors for *Escherichia coli* verotoxin (15–17). In addition, sphingolipids are becoming recognized as significant players in the control of cell growth, differentiation, and response to stress through the second messenger action of sphingolipid metabolites sphingosine, sphingosine-1-phosphate, and ceramide (18–20). Despite their importance, numerous gaps remain in the knowledge of sphingolipid metabolism, including the nature of the enzymes directly responsible for phytoceramide or ceramide formation from the presumed immediate precursor dihydroceramide (Fig. 1) (21).

In this report we present evidence that *S. cerevisiae* SYR2 is required for 4-hydroxylation of sphingoid bases and that this activity is necessary for syringomycin E action. We show that strains mutant in SYR2 produce sphingolipids missing the hydroxy group at the C-4 position of the long chain base moiety, that supplying such cells with C-4 hydroxylated long chain base suppresses the syringomycin E-resistant phenotype of syr2 strains, and that strains that overexpress Syr2p are enriched in 4-hydroxylase activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Yeast strains used in this study are listed in Table I. Yeast were grown at 28–30 °C with shaking. YPD, SC-ura, SG-ura (as SC-ura with glucose replaced by galactose), and SC-trp were as described by Kaiser et al. (22). The lcb1 mutants were grown in modified YPD (1% yeast extract, 1% peptone, 4% glucose, 50 mM sodium succinate, pH 5.0, 0.05% Tergitol (U. S. Biochemical...
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**FIG. 1. Sphingolipid biosynthesis in S. cerevisiae.** Shown is the likely pathway for *de novo* sphingolipid biosynthesis in yeast, along with the genes, known, involved at each step. The two possible pathways for long chain base 4-hydroxylation, proposed to be catalyzed by Syr2p, and long chain base acylation are shown. Hydroxylation of the very long fatty acid chain of 24 and 26 carbon length and very long fatty acid chain is thought to occur at some point after acylation. Very long fatty acid chains of 24 and 26 carbon length and long chain base acylation are shown. Hydroxylation of the pathways for long chain base 4-hydroxylation, proposed to be catalyzed by Syr2p, and long chain base acylation are shown. The abbreviations used are: PHS, phytosphingosine; DHS, dihydrosphingosine; IPC, inositolphosphorylceramide; MIPC, mannosylinositolphosphorylceramide; M(IP)2C, mannosyl-diinositolphosphorylceramide; PI, phosphatidylinositol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

The abbreviations used are: PHS, phytosphingosine; DHS, dihydrosphingosine; HPLC, high performance liquid chromatography; IPC, inositolphosphorylceramide; MIPC, mannosylinositolphosphorylceramide; M(IP)2C, mannosyl-diinositolphosphorylceramide; PI, phosphatidylinositol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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New England Nuclear Co., 20 mCi/μmol or 0.5 mCi of [4,5-3H]DHS (2.56 mCi/μmol) derived by hydrolysis of [3H]-N-acytely-DHS prepared as described previously (26). After culture for 18 h at 30 °C the cells reached a density of 1.9–2.4 × 10^6 cells/ml, and the reaction was stopped by adding trichloroacetic acid to a final concentration of 5%. The cells were processed to deacylate the ester lipids followed by extraction of the sphingolipids as described previously (27). To further purify the acidic sphingolipids, the sphingolipid extract was bound to AG4 resin (Bio-Rad) as described previously (28). The AG4 eluate was dried, dissolved in 1 ml of chloroform/methanol/water (16:15:3, v/v/v), and 3–7 μ1 aliquots were subjected to thin layer chromatography on 20-cm silica gel plates (Whatman HPI-K) with the solvent chloroform/methanol/4 N aqueous NH₄OH (9:7:2, v/v/v). Each lane contained a mixture of yeast PHS-containing sphingolipids: 2 nmol each of inositolphosphorylceramide (ICP) and mannosylinositolphosphorylceramide (MIPC) species containing mono- and di-OH fatty acids and mannosyl-diinositolphosphorylceramide (M(IP)2C) with a mono-OH fatty acid. Radioactivity was measured with a BioScan apparatus. The standards were located by charring after spraying with 10% (w/v) CuSO₄·5H₂O in 8% H₃PO₄ followed by heating at 160 °C for 30 min (29). The mannosylated sphingolipids in the deacylated lipid extract were also detected after thin layer chromatography of larger aliquots (125 μl) on 20-cm Whatman K3 plates developed with the same solvent as above. The plate was first treated with orcinol reagent (30) to detect the carbohydrate containing sphingolipids, MIPC and M(IP)₂C, and then treated with the CuSO₄/phosphoric acid reagent. A new band formed below MIPC and M(IP)₂C bands representing the free fatty acid portion of the sphingolipids.

To examine the nature of the long chain bases in the sphingolipid fractions, a portion of the [4,5-3H]DHS-labeled AG4 eluates were dried and hydrolyzed in 1 N HCl in methanol/water (82:18) at 80 °C for 18 h. The hydrolysates were dried, dissolved in chloroform/methanol/water (16:15:3, v/v/v), and spotted on Whatman K3 plates along with 20 nmol of DHS and DHS standards in each lane. The plates were developed with chloroform/methanol/2 N aqueous NH₄OH (40:10:1). Radioactivity was measured with a BioScan apparatus followed by detection of the standards with ninhydrin reagent.

**Fatty Acid Analysis—**Fatty acids from an acidic sphingolipid fraction (prepared as described above without the addition of radioisotopes) or whole cells were liberated by saponification and converted to UV-absorbing phenacyl derivatives that were resolved by reverse phase HPLC as described previously (25).

**Assay of 4-Hydroxyase Activity—**The previously constructed SYP2 overexpression plasmid, pYSYP2, placed a 5′-truncated SYP2 gene under the control of the galactose-inducible promoter GAL1 (11). For this work the truncated SYP2 insert was removed and replaced with an AUS1 deletion fragment containing the entire SYP2 coding region. Expression of Syr2p from this construct, pYSYP2a, was confirmed by observation of galactose-inducible complementation of syringomycin E resistance of a syc2 strain.

W303C containing pYSYP2 or the control plasmid pYES2 was grown overnight in SC-ura. Cells were washed with sterile water and diluted into 300 ml of SG-ura (8 × 10⁵ cells/ml) to initiate Syr2p expression. Cells were harvested after 16 h of growth and washed with water. W303C cells were grown similarly except the medium was SC-ura at each step. Microsome preparation was modified from published procedures (31). The washed cell pellet was resuspended in 25 ml of 100 mM Tris-sulfate, pH 9.4, 10 mM dithiothreitol and incubated at room temperature 15 min, followed by a wash with 10 mM Tris-HCl, pH 7.5, 0.6 M sorbitol, 0.1 mM diethiothreitol, 0.1 mM EDTA. Cells were then incubated 1 h at 30 °C in 7.5 ml of 10 mM Tris-HCl, pH 7.5, 2 mM sorbitol, 0.1 mM diethiothreitol, 0.1 mM EDTA, 0.1 mg/ml Zymolyase 100T (Seikagaku Corp., Tokyo). After washing with 10 mM Tris-HCl, pH 7.5, 2 mM sorbitol, cells were disrupted with glass beads in 1 ml of cold 10 mM Tris-HCl, pH 7.5, 0.65 mM sorbitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin. Glass beads and cell debris were removed by centrifugation. Microsomal membranes were collected by centrifugation at 4 °C for 90 min at 100,000 × g. Pellets were homogenized in 0.6 ml of cold 10 mM Tris, pH 7.5, 20% glycerol. Protein concentrations of the microsomal preparations were determined using Pierce Coomassie protein assay reagent with bovine serum albumin as standard.

To assay 4-hydroxylase activity, a control of either DHS or dihydrosphingosine in chloroform was dried in a stream of nitrogen and then resuspended by sonication in 0.1 ml 0.3% CHAPS. This was combined with 0.1 ml of 100 mM Tris-HCl, pH 7.5, 0.2 mM NADPH, 0.2 mM NADH, and microsomes (0.7 mg of protein) to initiate the reaction. Incubation was at 25 °C for 90 min, followed by methanol-HCl hydrolysis, 4-biphenylcarboxylate derivatization, and reverse phase HPLC analysis of long chain bases as described above. Chromatograms were inte...
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RESULTS

Sequence Analysis Suggests That Syr2p Is a Diiron Binding Lipid Hydroxylase or Desaturase—BLAST algorithm (34) comparisons of the deduced amino acid sequence of Syr2p with those in protein data bases showed significant similarities to endoplasmic reticulum proteins associated with lipid metabolism. In particular, close similarities were found with Arabidopsis thaliana ERG3 (C-5 stereol desaturase, score 51, p = 0.0098, 33% identities, 56% positives), Arabidopsis thaliana ERG3 (C-5 stereol desaturase, score 77, p = 0.014, 44% identities, 68% positives), and yeast ERG25 (C-4 sterol methyl oxidase, score 65, p = 4.5 x 10^-6, 52% identities, 65% positives). Similar findings were reported by Bard et al. (35) and Li and Kaplan (36). Despite the similarity of Syr2p to enzymes of sterol biosynthesis, an involvement for Syr2p in sterol metabolism could not be found. Comparisons of sterol profiles of syr2 mutants with similarly grown wild type strains revealed no differences (11), indicating that Syr2p functions in some other metabolic pathway.

Sequence comparisons also pointed to the occurrence in Syr2p of an eight-histidine motif grouped into three characteristic clusters (Fig. 2). Shanklin et al. (37) have recently reported that 75 proteins of known function contain this eight-histidine motif, which is thought to bind a catalytically active diiron cluster. Of these proteins, 66 are integral membrane proteins. All 66 catalyze 1 of 11 distinct O2-dependent modifications of hydrocarbon substrates, acting as either desaturases, hydroxylases, oxidases, or decarbonylases (37). Syr2p contains the eight-histidine motif and, based on sequence analysis and subcellular fractionation studies, is an integral membrane protein (11) and thus was predicted to also catalyze an O2-dependent modification of a hydrocarbon substrate. Because SYR2 did not appear to play a role in sterol biosynthesis and also differed from the yeast gene, OLE1, required for glyceride fatty acid desaturation (38), we were prompted to investigate the potential involvement of SYR2 in sphingolipid synthesis. In the yeast sphingolipid biosynthetic pathway (Fig. 1), two uncharacterized processes were deemed potential candidates for catalysis by eight-histidine motif hydroxylases: C-4 hydroxylation of the sphingoid long chain base portion of the sphingolipid and hydroxylation of the very long chain fatty acid.

Yeast Strains Deficient in SYR2 Lack the Sphingoid Long Chain Base Phytosphingosine—Sphingolipids in yeast are normally composed of the sphingoid long chain base PHS (N-4-hydroxyphinganine), typically of 18 or 20 carbon chain length (C-18 and C-20, respectively), an amide-linked very long chain fatty acid, primarily mono-hydroxy-C-26 chains with lesser amounts of di- and nonhydroxyl forms, and a phosphinositol-containing head group (39) (Fig. 1). To test the involvement of Syr2p in the sphingoid base 4-hydroxylation, the sphingoid base compositions of mutant strain WΔsyr2Δ and the isogenic wild type strain W303C were determined (25). Reverse phase HPLC separation of biphenylcarbonyl-derivatized long chain bases derived from the wild type strain W303C revealed two peaks of UV-absorbing material, as expected, with retention times of 15 and 25 min (Fig. 3B). Coelution with a derivatized C-18 PHS standard and electrospray mass spectral analysis of the material collected from the two peaks verified their identities as C-18 PHS at 15 min and mainly C-20 PHS at 25 min. The derivatized long chain bases from the Δsyr2 mutant strain also separated into two peaks, with one again eluting at 25 min but the other eluting at 42 min (Fig. 3C). Little material with a retention time of 15 min was apparent (<0.3% of total long chain base). Authentic C-18 PHS treated in the same manner as the lipid extracts eluted with a retention time of 25 min (Fig. 3A). It was not possible to distinguish between the C-20 PHS and C-18 DHS derivatives by the chromatography system used, but mass spectral analysis of the Δsyr2 material eluting at 25 min revealed a molecular ion mass of 482.3 Da, consistent with its assignment as the N-biphenylcarbonyl derivative of C-18 DHS. The mass of the material eluting at 42 min was 510.4 Da, as expected for N-biphenylcarbonyl-C-20 DHS. Similar results were obtained with an independently isolated syr2 mutant strain in a different genetic background (13N-F2 (syr2) and KZ1–1C (SYR2); data not shown). Thin layer chromatography of acid-hydrolyzed, radiolabeled...
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SYR2 S. c. (173–199 / 247–276) YPLR4LMNKKYTLYKFSVBSVHHELVP / DDCGCYALPLDPEQ–WLPFNAYVHED1HQQ
ERG3 S. c. (198–223 / 269–294) YLAKHRWSWPD–YKALHKHKKWLC / MIQKQQMSNNNPW-----VWNGATCTVHLY
ERG3 A. t. (144–170 / 219–244) YWNRRLHDKPLYKLVABTTHYNKQ / N1KDCIQNHNPW-------VAMAGYHT1BT
SYR25 S. c. (157–182 / 234–265) YWAHRFHYG–VFYKVIHKHHRYAAP / DQSGIDFPWSLKINMF PWAGAEHHD1HHY

FIG. 2. Putative metal binding domains of Syr2p and similarity to sterol biosynthetic enzymes. The sequences were obtained as part of a BLAST analysis and are from S. cerevisiae (S.c.) or A. thaliana (A.t.). Histidine residues identified by Shanklin et al. (58) as being involved in metal binding are indicated in bold type. Histidine residues identified by Li and Kaplan (36) as a fourth histidine cluster motif specific to the ERG3/ERG25/SYR2 family of sequences are underlined.

FIG. 3. Sphingoid long chain bases produced by wild type and Δsyr2 strains. Methanol-HCl hydrolysates of wild type strain W303 (B) and Δsyr2 cells (C), as well as C-18 DHS and C-18 PHS standards (A), were derivatized with the UV-absorbing 4-biphenylylcarbonyl chloride and resolved by reverse phase HPLC as described under “Experimental Procedures.” Effluent was monitored by absorbance at 280 nm.

sphingolipids from Δsyr2 and wild type cells confirmed that the wild type produced sphingolipids containing primarily PHS (85%) with 15% DHS, whereas the Δsyr2 mutant produced solely DHS (data not shown). We conclude from these data that Syr2p is necessary for the 4-hydroxylation of the DHS component of yeast sphingolipids. These results are consistent with Syr2p functioning as dihydroceramide or DHS hydroxylase.

Fatty Acid Analysis of Δsyr2 Strain—The effect of the syr2 mutation on hydroxylation of the very long chain fatty acid component of sphingolipids was also examined as described under “Experimental Procedures.” Hydroxylation of the very long chain fatty acids was observed in the absence of Syr2p activity. For example, the percentage of distribution of non-, mono-, and di-hydroxyl fatty acids for wild type cells was 8, 78, and 14%, respectively, and for Δsyr2 cells, it was 53, 47, and 0%, respectively. Clearly Δsyr2 cells hydroxylate the very long chain fatty acid in sphingolipids. Why the distribution of hydroxylated species differs from wild type is not known.

In Vitro Measurement of 4-Hydroxylase Activity—Sphingoid base 4-hydroxylase activity was measured in microsomal fractions, because Syr2p has previously been localized to the endoplasmic reticulum (11). Microsomes, prepared from SYR2 wild type, overexpression, and deletion strains, were supplied with substrate, either DHS or dihydroceramide solubilized in CHAPS, along with NADH and NADPH. Both NADH and NADPH have been reported to be cofactors for activity of other putative diiron proteins involved in oxygen-dependent reactions of hydrocarbon substrates. After 90 min at 25 °C, sphingoid long chain bases were released by methanol-HCl hydrolysis and extracted, and their 4-biphenylylcarbonyl derivatives were separated and quantitated by reverse phase HPLC. Hydroxylated product was apparent when DHS or dihydroceramide were supplied to microsomes from a SYR2 overexpressing strain, W303C(pYES2a). Using the same amount of protein, 3–4-fold less hydroxylated product was produced if NADH and NADPH were omitted from the reaction or if the source of NADPH was a wild type strain, W303C(pYES2), containing only the chromosomal copy of SYR2 and a control plasmid (Table II). No 4-hydroxyl products were detected if microsomes were from the deletion strain WΔsyr2a.

Growth in the Presence of PHS Suppresses the syr2 Phenotype—To confirm that the syringomycin E resistance phenotype of the syr2 mutant is due to a loss of hydroxylase activity rather than an additional unknown activity of Syr2p, we wished to test whether syringomycin E sensitivity is restored by supplying syr2 cells with the product of the hydroxylase. It is still uncertain, however, if the hydroxylase substrate is phytoceramide, PHS, or both (see “Discussion”). As yeast do not readily utilize exogenous ceramides but will incorporate exogenous sphingoid long chain bases into the sphingolipid biosynthetic pathway (40), syringomycin E sensitivity was tested following growth in medium containing hydroxylated (PHS) or nonhydroxylated (DHS) long chain bases rather than hydroxylated or nonhydroxylated ceramides. To achieve this, the lcb1 gene was deleted in strain WΔsyr2a (see “Experimental Procedures”). LCB1 encodes a subunit of serine palmitoyl transferase, the first enzyme in sphingolipid biosynthesis (Fig. 1). An lcb1 mutation results in auxotrophy for long chain bases (32).

The Δsyr2 Δlcb1 double mutant was grown several generations in medium containing either DHS or PHS to ensure that sphingolipids lacked or contained phytoceramide, respectively. Each culture was diluted into fresh medium and then challenged with increasing concentrations of syringomycin E. The effectiveness of syringomycin E was assessed after an additional 16 h of growth. For the Δsyr2 Δlcb1 strain, syringomycin E had no noticeable effect on growth of the DHS-containing cultures. The PHS-containing cultures, on the other hand, showed essentially no growth at syringomycin E concentrations of 1 µg/ml and higher (Fig. 4). These results, with the Δsyr2 Δlcb1 strain supplemented with either DHS or PHS, mimicked those obtained upon syringomycin E treatment of Δsyr2 and SYR2 strains, respectively (Fig. 4). Further, an isogenic ΔSYR2 Δlcb1 strain, which retains Syr2p function, also retained its syringomycin E sensitivity whether supplied with DHS or PHS in the growth medium (not shown). Thus, the Δsyr2 Δlcb1 strain was restored to the wild type syringomycin E-sensitive phenotype by supplying it with hydroxylated long chain base, and the difference between Δsyr2 Δlcb1 cultures grown on PHS or DHS was dependent on a lack of Syr2p function. These
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Sphingolipid Analysis of Δsyr2 Strain—The fact that Δsyr2 mutants are viable, whereas sphingolipids are essential, raised serious questions about the mutant lipid composition. The loss of long chain base hydroxylation could result in the simple substitution of nonhydroxylated long chain base for 4-hydroxy-long chain base or, alternatively, in an alteration of the overall levels or types of sphingolipids. The fact that Δsyr2 Strains are viable, whereas sphingolipids are essential, raised serious questions about the mutant lipid composition. The loss of long chain base hydroxylation could result in the simple substitution of nonhydroxylated long chain base for 4-hydroxy-long chain base or, alternatively, in an alteration of the overall levels or types of sphingolipids. The recovery of similar quantities of long chain base from mutant and wild type strains (Fig. 3) would argue against a large alteration of the overall levels or types of sphingolipids. The fact that Δsyr2 mutants are viable, whereas sphingolipids are essential, raised serious questions about the mutant lipid composition. The loss of long chain base hydroxylation could result in the simple substitution of nonhydroxylated long chain base for 4-hydroxy-long chain base or, alternatively, in an alteration of the overall levels or types of sphingolipids. The recovery of similar quantities of long chain base from mutant and wild type strains (Fig. 3) would argue against a large alteration of the overall levels or types of sphingolipids.

![Figure 3](image3.png)

**FIG. 3.** Thin layer chromatography of sphingolipids obtained by in vivo labeling of wild type and Δsyr2 cells with [3H]DHS. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standard were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring. Wild type W303C and Δsyr2 cells were grown in the presence of [4,5-3H]DHS and then processed to deacylate the sphingolipids. The standards were located by charring.
5 consists of inositol-labeled sphingolipids. Second, as stated above, analysis of the HCl-methanol hydrolysate of the sphingo-
lipid preparation from the mutant strain exhibits only DHS, not PHS, whereas the wild type shows mainly PHS and some
DHS. Finally, when the sphingolipid preparations from unla-
beled cells are subjected to thin layer chromatography followed
by staining for mannose by spraying with orcinol-sulfuric acid,
the mutant exhibits orcinol positive lipids in the locations of
species C and D; species E and F, which are located at higher
Rf values than the orcinol-positive spots in wild type cells;
species I and J (MIPC); and species K (M(IP6)2Cs). This result
is consistent with the conclusions that species C and D and
species E and F are MIPC and M(IP6)2Cs species, respectively.
We conclude that the Δsyr2 cells make sphingolipids with head
groups similar to those found in wild type cells but that their
ceramide moieties contain only DHS and no PHS.

DISCUSSION

The present study of yeast gene SYR2 reveals that sphingo-
lipids play a key role in the action of the antifungal syringo-
mycin E. It also uncovers a previously uncharacterized activity
of the sphingolipid biosynthetic pathway. The lack of PHS-
based sphingolipids and accumulation of DHS-based sphingo-
lipids in syringomycin E-resistant syr2 mutants, the greater
4-hydroxylase activity in a SYR2 overexpression strain, the lack
of activity in a syr2 mutant strain, and the ability to bypass
the syr2 defect with exogenously added PHS provide support
for a biosynthetic role of Syr2p in 4-hydroxylation of
spingoid bases. In addition, the sequence similarity of Syr2p
to membrane hydroxylases and desaturases and its localization
in the endoplasmic reticulum (11), the site of early sphingolipid
biosynthetic steps (41), suggest that Syr2p is the enzyme that
catalyzes this specific hydroxylation step. An essential cata-
ytic role in fatty acid hydroxylation is not likely because very
long chain fatty acid hydroxylation was still detected in the
syr2 mutant despite a complete loss of SYR2 transcripts (11).
While this report was in preparation, Haak et al. (42) reported
that an independent sur2Δsyr2 mutant produces sphingolipids
lacking long chain base 4-hydroxylation, whereas a second gene
product, Sca7p, is required for sphingolipid very long chain
fatty acid hydroxylation.

Identification of Syr2p as the sphingolipid 4-hydroxylase will
pave the way for isolation and characterization of the enzyme.
This will, in turn, permit clarification of several issues regard-
ing sphingolipid biosynthesis. One important question con-
cerns the identity of the lipid substrate of Syr2p. The analogous
reaction in animal cells, desaturation at the C-4 position, is
thought to occur at the level of dihydroceramide (43). In yeast,
however, it is not known if 4-hydroxylation occurs before or
after long chain base acylation, i.e. if Syr2p converts DHS to
PHS, dihydroceramide to phytoceramide, or both (Fig. 1). In-
hibition of ceramide synthesis leads to accumulation of both
DHS and PHS (44, 45), suggesting a direct conversion from
DHS to PHS is possible, at least under conditions of limited
ceramide formation. We have shown that incubation of either
sphingolipid with acyltransferase before hydroxylation
leads to hydroxylation. With crude microsomal preparations,
however, enzymes capable of catalyzing acylation or decylation of the added substrate before hydroxy-
lation may be present and obscure the true nature of the
substrate.

Confirmation that Syr2p is in fact an iron-containing oxida-
tive enzyme as predicted from the sequence (Fig. 2) will also be
afforded by biochemical analysis. The stimulation of 4-hydroxy-
lipase activity by reduced pyridine nucleotide, as shown here,
is consistent with Syr2p being a member of this enzyme family.
Molecular oxygen has been shown to be the main source of
oxygen added to dihydroceramide (46), but little else is known
of the mechanism of this reaction. Knowledge about Syr2p will
also reveal mechanisms about mammalian sphingolipid bio-
synthesis. Phytoceramide is produced by certain mammalian
tissues (47) as well as by fungi and plants, and these tissues are
predicted to contain a Syr2p homolog. The primary mammalian
sphingolipid-based ceramide contain the long chain base
sphingosine, which has a C-4,5 double bond rather than the
4-hydroxy group. Recent reports concerning the enzyme in rats
that catalyzes this reaction, dihydroceramide desaturase, sug-
gest it also has properties similar to diiron-containing lipid
desaturases and hydroxylases (43, 48).

How sphingolipids, and more specifically sphingolipid 4-hy-
droxylation, allow susceptibility to syringomycin E can only be
speculated. One possibility is that 4-hydroxylated sphingolip-
ids directly bind this antifungal compound at the cell surface.
The 4-hydroxy group is expected to influence the degree of
sphingolipid exposure on the membrane surface, but it will also
affect lipid and protein nearest neighbor interactions in the
plane of the membrane. Another possibility is that 4-hydroxyl-
ated sphingolipids indirectly influence syringomycin E-cell in-
teraction by modulating sterol or glycerophospholipid composi-
tions or both. Syringomycin E action is influenced by sterols (8,
10), phospholipid bilayers facilitate ion channel formation by
syringomycin E molecules (49), and syr2 mutants have lowered
cellular glycerophospholipid levels (11). Despite evidence for
cross-regulation of the biosynthetic pathways of these various
lipid classes in yeast (50), it is difficult to predict precisely how
an alteration in the hydroxylation state of sphingolipids could
influence cellular sterol and phospholipid composition. Fur-
thermore, 4-hydroxylation could influence the insertion and
assembly of lipids as well as proteins into the plasma mem-
brane. Finally, the requirement for sphingolipid 4-hydroxy-
al action may reflect the involvement of phytoceramide-mediated
growth inhibition processes in syringomycin E action. Phyto-
ceramide and ceramide, but not dihydroceramide, are reported
to mediate cell death in animal cells and growth inhibition in
yeast (18, 51, 52), although the phenomenon is not always
observed (53). Exposure of yeast cells to syringomycin E may
cause increased cellular levels of phytoceramide (perhaps by
activating sphingolipid turnover), which in turn may activate
specific protein kinases and phosphatases (18, 51, 52), leading
to growth arrest. Without the ability to hydroxylate dihydro-
ceramide to phytoceramide and the consequent substitution of
dihydroceramide into mature sphingolipids, syr2 mutants
would be incapable of undergoing this process.

The observation that SYR2 encodes a nonessential function
raises questions about the cellular roles of 4-hydroxylated
sphingolipids in yeast growth and survival. Normal SYR2
strains produce sphingolipids that are based almost exclusively
on phytoceramide (this study and Ref. 54), but syr2 mutants
grow well with dihydroceramide-based sphingolipids. Sphingo-
lipids are indicated to be required for maintaining proton per-
meability barriers across the membrane or for proton extrusion
(55) and for maturation of glycosylphosphatidylinositol-ant
proteins (56). Preliminary observations, however, show
that syr2 mutants display wild type growth phenotypes under
conditions where proper functioning of these processes may be
essential, namely at acidic pHs (4.1), high temperatures
(39 °C), and high salt concentrations (0.75 M NaCl).3 Also,
Calcofluor staining of chitin, a probe of cell wall structure, was
unperturbed in the syr2 mutant, although growth of the syr2
mutant was slightly retarded by Calcofluor.4 The two pheno-

3 J. Y. Takemoto, unpublished results.
4 M. M. Grilley and J. Y. Takemoto, unpublished results.
types previously associated with syr2/sur2 mutations, resistance to syringomycin E, and suppression of rvs161 mutations can now be said to be associated with a loss of 4-hydroxylation of the long chain base moiety of sphingolipids. The only apparent commonality of these two phenotypes is growth restoration under conditions that inhibit growth of wild type cells. The mechanism(s) responsible for these effects await elucidation, as does a clear definition of the role of 4-hydroxy-sphingolipids in yeast biology and syringomycin E action.

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REFERENCES

1. Takemoto, J. Y. (1992) in Bacterial Phytotoxin Syringomycin and Its Interaction with Host Membranes, ed. by Gross, D. (1992) Annu. Rev. Phytopathol. 29, 247–278
2. Hrabak, E. M., and Willis, D. K. (1993) Mol. Plant-Microbe Interact. 6, 368–375
3. Adefuyi, F. C., Isogai, A., Giorgio, D. D., Ballio, A., and Takemoto, J. Y. (1995) FEBS Lett. 373, 63–67
4. Hutchinson, M. L., Tester, M. A., and Gross, D. C. (1995) Mol. Plant-Microbe Interact. 8, 610–620
5. Zhang, L., and Takemoto, J. Y. (1987) Phytopathology 77, 297–303
6. Takemoto, J. Y., Yu, Y., Stock, S. D., and Miyakawa, T. (1993) FEBS Lett. 314, 339–342
7. Taguchi, N., Takano, Y., Julmanop, C., Wang, Y., Stock, S., Takemoto, J., and Miyakawa, T. (1994) Microbiology 140, 353–359
8. Julmanop, C., Takano, Y., Julmanop, C., Wang, Y., Stock, S., Takemoto, J., and Miyakawa, T. (1994) Microbiology 140, 353–359
9. van der Rest, M. E., Kamminga, A. H., Nakano, A., Arakaki, Y., Poolman, B., and Konings, W. N. (1995) Microbiol. Res. 99, 304–322
10. Hannun, Y. A., and Bell, R. M. (1989) Science 243, 500–507
11. Fishman, P. H., Pauschuk, T., and Orlandi, P. A. (1993) Adv. Lipid Res. 25, 165–187
12. Lingwood, C. A. (1991) Adv. Lipid Res. 25, 189–211
13. Lingwood, C. A. (1996) Trends Microbiol. 4, 147–153
14. Hannun, Y. A. (1996) Science 274, 1855–1859
15. Dickson, R. C., Nagiec, E. E., Skrzypek, M., Tillman, P., Wells, G. B., and Lester, R. L. (1997) J. Biol. Chem. 272, 30196–30200
16. Jenkins, G. M., Richards, A., Wahl, T., Mao, C., Obeid, L., and Hannun, Y. A. (1996) J. Biol. Chem. 272, 30196–30200
17. Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garrett, S., and Hannun, Y. A. (1996) J. Membr. Biol. 149, 41–47
18. Greenberg, M. L., and Lopes, J. M. (1996) Microbiol. Rev. 60, 1–20
19. Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garrett, S., and Hannun, Y. A. (1996) J. Biol. Chem. 272, 9255–9261
20. Nickels, J. T., and Broach, J. R. (1996) Genes Dev. 10, 382–394
21. Ells, K. M., Qi, C., Dolan, J. W., Thompson, R. P., and Meier, K. E. (1997) Arch. Biochem. Biophys. 340, 101–110
22. Smith, S. W., and Lester, R. L. (1974) J. Biol. Chem. 249, 3395–3405
23. Bukowski, J. E., Bang, J. M., and Kim, D. Y. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 183–190
24. Skrzypek, M., Lester, R. L., and Dickson, R. C. (1993) J. Biol. Chem. 268, 845–850
25. Fewster, M. E., Burns, B. N., and Mead, J. F. (1969) J. Chromatogr. 43, 120–126
26. Skrifvars, V. P., and Bronckers, M. (1969) Methods Enzymol. 14, 545–546
27. Kato, R., Yasumori, T., and Yamazoe, Y. (1991) Methods Enzymol. 206, 183–190
28. Adetuyi, F. C., Isogai, A., Giorgio, D. D., Ballio, A., and Takemoto, J. Y. (1995) Mol. Plant-Microbe Interact. 8, 2176–2181
29. Nagiec, M. M., Nagiec, E. E., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1997) J. Biol. Chem. 272, 9809–9817
30. Zweerink, M. M., Edison, A. M., Wells, G. B., Pinto, W., and Lester, R. L. (1992) J. Biol. Chem. 267, 25032–25038
31. Lester, R. L., Wells, G. B., Oxford, G., and Dickson, R. C. (1993) J. Biol. Chem. 268(24), 24957–24960
32. Bueso, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L., and Dickson, R. C. (1993) J. Biol. Chem. 268(24), 24957–24960