Identification of a *Saccharomyces* Gene, *LCB3*, Necessary for Incorporation of Exogenous Long Chain Bases into Sphingolipids*

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To identify genes necessary for sphingolipid synthesis in *Saccharomyces cerevisiae* we developed a procedure to enrich for mutants unable to incorporate exogenous long chain base into sphingolipids. We show here that a mutant strain, AG84-3, isolated by using the enrichment procedure, makes sphingolipids from endogenously synthesized but not from exogenously supplied long chain base. A gene termed *LCB3* (YJL134W, GenBank designation X87371x21), which complements the long chain base utilization defect of strain AG84-3, was isolated from a genomic DNA library. The gene is predicted to encode a protein with multiple membrane-spanning domains and a COOH-terminal glycosylphosphatidylinositol cleavage/attachment site. Deletion of the *lcb3* gene in a wild type genetic background reduces the rate of exogenous long chain base incorporation into sphingolipids and makes the host strain more resistant to growth inhibition by long chain bases. Only one protein in current data bases, the *S. cerevisiae* open-reading frame YKR053C, whose function is unknown, shows homology to the Lcb3 protein. The two proteins are not, however, functional homologs because deletion of the YKR053C open reading frame does not impair long chain base utilization or enhance resistance of cells to growth inhibition by long chain bases. Based upon these data we hypothesize that the Lcb3 protein is a plasma membrane transporter capable of transporting sphingoid long chain bases into cells. It is the first candidate for such a transporter and the first member of what appears to be a new class of membrane-bound proteins.

Sphingolipids comprise a diverse group of complex lipids found primarily in the plasma membrane of all known eukaryotes. Knowledge of the structures and functions of sphingolipids has expanded at an increasing rate, but much less is known about the enzymes and genes needed for synthesis of sphingolipids. In one approach to identify sphingolipid biosynthetic genes, our laboratory devised a unique procedure for isolating mutants of *Saccharomyces cerevisiae* defective in sphingolipid synthesis. As described here, this approach led to the isolation and characterization of a strain defective in the incorporation of exogenous sphingoid long chain bases into sphingolipids.

Synthesis of the long chain base component of sphingolipids (Fig. 1) begins with the condensation of serine and palmitoyl-CoA to yield 3-ketosphinganine. This essentially irreversible reaction is catalyzed by serine palmitoyltransferase (3-ketosphinganine synthase (EC 2.3.1.50); for review, see Ref. 1), encoded by the *LCB1* (2) and *LCB2* (3) genes. The 3-ketosphinganine is converted to the long chain base sphinganine (dihydrosphingosine) which is N-fatty-acylated to yield dihydroceramide. Dehydrogenation of dihydroceramide in animals yields ceramide containing the long chain base sphingosine, which is rapidly converted to sphingolipids by the addition of polar components to the 1-hydroxyl group. The predominant ceramides in fungi and plants contain N-α-hydroxy fatty-acylphytosphingosine (4) formed by undefined hydroxylation reactions. Phytosphingosine (PHS) lacks the 4,5-double bond found in sphingosine and has instead an hydroxyl group at the 4 position (Fig. 1).

In *S. cerevisiae* and other fungi the 1-hydroxyl of phytoceramide is modified by addition of myo-inositol phosphate to form IPC which is then mannosylated to yield mannose-inositol-P-ceramide (MIPC). The final step in *S. cerevisiae* sphingolipid synthesis is the addition of inositol-P to MIPC to yield the major sphingolipid mannose-(inositol-P)2-ceramide (M(IP)2C; Refs. 5–7).

We describe here a rationale and procedure for isolating mutants defective in synthesis of intermediates formed between 3-ketosphinganine and MIPC (Fig. 1). The procedure was designed to avoid the potential problems of growth inhibition and killing of cells by the buildup of a sphingolipid intermediate. It is known that exogenous N-acetyl-sphingosine (C2-ceramide) inhibits growth of *S. cerevisiae* cells (8, 9), as does exogenous PHS. Growth inhibition and killing were avoided by using a sphingolipid compensatory (SLC) strain that can grow without making sphingolipids, unlike normal yeast strains that require sphingolipids for growth and viability (10). SLC strain 7R6 carries two mutations, a deletion of the *LCB1* gene and a point mutation that creates the suppressor gene *SLC1-1* (11). In the absence of exogenous long chain base, the suppressor gene causes cells to make novel glycerophospholipids that compensate for one or more functions of sphingolipids essential for vegetative growth (12). The *lcbl* mutation blocks the first committed step in sphingolipid synthesis, so no toxic sphingolipid intermediate should accumulate when cells are grown on medium lacking a long chain base. Thus if an SLC strain becomes mutated in, for example, the ceramide synthase

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This paper is dedicated to the memory of Bharath Srinivasan (deceased 1990) who isolated the original AG84 strain and found its growth inhibition by long chain bases. Only one protein in current data bases, the *S. cerevisiae* open-reading frame YKR053C, whose function is unknown, shows homology to the Lcb3 protein. The two proteins are not, however, functional homologs because deletion of the YKR053C open reading frame does not impair long chain base utilization or enhance resistance of cells to growth inhibition by long chain bases. Based upon these data we hypothesize that the Lcb3 protein is a plasma membrane transporter capable of transporting sphingoid long chain bases into cells. It is the first candidate for such a transporter and the first member of what appears to be a new class of membrane-bound proteins.

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1 The abbreviations used are: PHS, phytosphingosine; IPC, inositol phosphoceramide; MIPC, mannose-inositol-P-ceramide; M(IP)2C, mannose-(inositol-P)2-ceramide; SLC, sphingolipid compensatory; kb, kilobase(s); PI, phosphatidylinositol.

2 M. Skrzypek, R. L. Lester, and R. C. Dickson, manuscript in preparation.
**Fig. 1. Pathway of sphingolipid synthesis in S. cerevisiae.** Known pathway intermediates, substrates, genes (LCB1 and LCB2), and enzymes are indicated (4). Undefined steps are in brackets or indicated by question marks.

Gene (Fig. 1) the mutant strain should continue to grow without making sphingolipids because of the SLC1-1 suppressor gene and should not accumulate a toxic long chain base intermediate because of the lcb1 mutation.

The mutant derivative of 7R6 described here has an impaired ability to incorporate exogenous long chain bases into sphingolipids because of a mutation in the LCB3 gene (long chain base). The Lcb3 protein is predicted to have several membrane-spanning domains, suggesting that it is a membrane-bound transporter or a facilitator of long chain base uptake. It is structurally but not functionally homologous to another yeast protein of unknown function. The Lcb3 protein is not homologous to any other predicted protein, thus it appears to be the first member of a new class of membrane-bound proteins with a potential transporter function.

**EXPERIMENTAL PROCEDURES**

S. cerevisiae strains used in these studies are described in Table I. Culture conditions have been described previously (13). Reagents hygromycin B, canavanine, DL-erythrosphinganine, stearylamine, and DL-threosphinganine were from Sigma. Cerulenin was from Makor Chemicals Ltd. Sphingofungin B was from Merck. N-Acetylsphingosine was from Matreya, Inc. D-Erythrosphingosine was from Serdary Research Laboratories. For phytosphingosine, see Ref. 14.

The LCB3 gene was isolated from a S. cerevisiae genomic DNA (strain 4R3, 10) library made by ligating Sau3AI-cut DNA fragments into the BamHI site of pRS15 (CEN6, LEU2, 15). The library was transformed (16) into strain AG84-3, and Leu" transformants were selected on minimal agar plates lacking leucine, buffered with 50 mM sodium succinate, pH 5.5, and incubated at 30°C for 10 days. About 15,000 transformants were tested by replica plating for colonies able to grow at 37°C on defined agar plates lacking leucine and sodium succinate but containing 25 μg PHS. These conditions permitted growth of the parental strain 7R6 but restricted growth of the mutant derivative AG84-3. One out of 15 colonies that grew under these conditions contained a plasmid, pJAB1-5, with a 6.5-kb insert, which, when isolated by transformation into Escherichia coli and retransformed into strain AG84-3, gave transformants able to grow at 37°C in the presence of PHS. The DNA sequence of a portion of the yeast insert was determined and compared with the GenBank data base using the BLAST algorithm (17). Predicted restriction sites were used to subclone portions of the insert, carrying the complementing activity, into pRS315, yielding a 2.4-kb SpeI-MscI DNA fragment containing only one large open reading frame, YDL34W. Other subclones failed to complement.

The lcb3-Δl deletion allele has the 522 base pairs between the BamHI and the NsiI sites of the LCB3 coding region replaced with pRS305 (LEU2, 15) to give MPRS305. MPRS305 was constructed by cloning a 3.1-kb PstI-BamHI DNA fragment from pJAB1-5, containing the promoter and 5′ end of the LCB3 gene, into pRS305 cut with the same restriction endonucleases. The resulting plasmid was cut with PstI and MscI and ligated with a 1-kb NsiI-MscI DNA fragment, containing the 3′-coding and flanking region of LCB3, obtained from pJAB1-5. Deletion of MscI gives a linear DNA fragment with ends homologous to the 5′- and 3′-noncoding region of LCB3. Yeast strains carrying the lcb3-Δl allele were made by transformation with this linear molecule followed by selection for Leu" cells. Replacement of LCB3 by the deletion allele was verified by Southern blotting. A wild-type allele of LCB3 was tagged at the HpaI site, located about 0.8 kb upstream of the LCB3 coding region, with the S. cerevisiae LEU2-selectable marker gene. The tagged allele was transformed into Leu" yeast cells as a 3.6-kb MscI DNA fragment with selection for Leu" cells.

Construction of pLCB1-5 began by inserting the S. cerevisiae ADE2 gene as a PstI-SpeI DNA fragment into pRS315 (15) cut with the same restriction endonucleases. The resulting plasmid was cut with NotI and SacI and ligated to the LCB1 gene obtained from pTZ18-LCB1 (2) as an NotI-SacI DNA fragment.

A 2.3-kb DNA fragment containing the YKR0653C open reading frame was made from genomic DNA by using the polymerase chain reaction and cloned into pRS315 as an EcoRI-PstI fragment to give pJAB2.

**Isolation of Strain AG84-3**

Strain 7R6 was grown overnight in PYED (1% yeast extract (Difco), 2% Bacto-Peptone (Difco), 2% glucose, 50 mM sodium succinate (pH 5.0), inositol (50 mg/ml), and potassium phosphate monobasic (0.5 g/liter)) plus 25 μg PHS (referred to here as the medium) and then incubated with ethylmethanesulfonate to give 20% killing (18). Mutagenized cells were diluted to an absorbance at 600 nm (A600) of 0.4 with medium, incubated with shaking at 30°C for 7 h, during which time the A600 increased to 2.5, centrifuged, resuspended in 2 ml of medium, and sonicated using a microtip (Heat Systems-Ultrasonic) for 2 min to disrupt clumped cells. One ml of cells was layered on 4 ml of 30% sodium diatrizoate (18) and centrifuged at 10°C in a Sorvall RT6000B centrifuge for 4 min at 2,000 rpm. Most cells were at the interface, but a faint pellet of dense cells was present at the bottom of the tube. The liquid was aspirated, and the cell pellet was carefully resuspended in 0.5 ml of medium to avoid mixing with cells stuck to the side of the tube. Resuspended pellets from two tubes were mixed and recentrifuged on 30% sodium diatrizoate. The cell pellet was resuspended in medium, and about 500 cells were spread on PYED plates containing 25 μg PHS. Two days later only about 15 colonies/plate were visible, suggesting that only 3% of the dense cells were viable.

**Measurement of Sphingolipid Synthesis in Vivo**

Protocol 1—Sphingolipid synthesis was measured by growing cells to saturation at 30°C in PYED, diluting to an A600 of 0.1 with fresh PYED medium containing 10 μg/ml of inositol and 10 μCi/ml myo-[2-3H]inositol (20 Ci/mmol, DuPont NEN), and grown at 30°C overnight to an A600 of 1. Cells were washed with 25 ml of 0.1 M sodium phosphate, pH 5.5, by centrifugation and resuspended in the same buffer at 50 A600 units/ml. A reaction containing 0.4 ml of 0.5% tergitol, 0.4 ml of 0.5 mM sodium succinate, pH 5.5, 0.1 ml of 40% glucose, and 10 μl of 2.5 mM PHS in 95% ethanol was initiated by addition of 0.2 ml of the radiolabeled cells. At time 0 a 0.2-ml sample and at the indicated times a 0.5-ml sample of radiolabeled cells were treated with trichloroacetic acid at a final concentration of 5%.
centration of 5% for 20 min at 0 °C. Cells were centrifuged, washed threem times with 1 ml of 5% trichloroacetic acid, and finally with water. Cell pellets were extracted with 1 ml of 95% ethanol, water, diethyl ether, pyridine, concentrated NH4OH (15:15:10:0:10:15:1, v/v) for 60 min at 60 °C (19). After centrifuging while warm, a 0.5-ml sample of the supernatant fluid was evaporated under a stream of N2, dissolved in 1 ml of monomethylamine reagent, and heated for 60 min at 52 °C to deacylate the acylester phospholipids (20). The sample was dried and dissolved in 0.5 ml of chloroform:methanol:water (16:16:5). Qualitative TLC of the samples was carried out by silica gel thin layer chromatography on 20-cm Whatman LK5 plates developed with chloroform, methanol, water (16:16:5). Radioactivity in the final band was measured by liquid scintillation spectrometry.

Uptake of Long Chain Bases

Cells were grown to an A600 of 0.6–0.8 at 30 °C in minimal medium, centrifuged, resuspended in medium lacking tergitol at an A600 of 5 units/ml, and diluted into 7.2 ml of minimal medium containing 0.056% tergitol and 11.11 μM [3H]phosphoglycerolamine (4,000 cpm/mmol) in a 50-ml plastic tube that was incubated with shaking at 30 °C. At various times duplicate 0.5-ml samples were diluted into 5 ml of ice-cold dilution buffer (0.02 N cyclohexirin (U. S. Biochemical Corp.), 0.05% tergitol, 10 μM phosphoglycerolamine), filtered (0.4-μm pore size, Nucleosep Corp., Pleasant, CA) to separate cells from the free radioisotope, and washed on the filter with 3 × 5 ml of ice-cold dilution buffer. Radioactivity on the filter was measured by liquid scintillation spectrometry.

RESULTS

Isolation of Strain AG84-3—Strain AG84-3 was isolated by using the procedure described under “Experimental Procedures” to enrich for strains defective in sphingolipid synthesis. Briefly, when SLC strains like 7R6 are fed PHS they make sphingolipids and have a normal buoyant density. If, however, a mutation has blocked sphingolipid synthesis the density of the cell should increase because sphingolipids will not be made in the presence of PHS. Using this strategy, SLC cells having a higher density following growth in the presence of PHS were enriched, and putative mutants were screened to differentiate those specifically defective in sphingolipid synthesis from those defective in other lipid biosynthetic pathways which might also affect cell density. The screen was based upon the observation that strain 7R6 cannot grow at low pH when it lacks sphingolipids (no PHS present in the medium) but can grow when allowed to make sphingolipids (PHS present in the medium) (22). Thus, about 100 mutants unable to grow on PYED plates at pH 4.1 either in the presence or absence of 25 μM PHS were identified.

Mutants specifically defective in sphingolipid synthesis rather than glycerophospholipid synthesis were identified by looking for decreased incorporation of [3H]inositol into inositol-containing sphingolipids relative to phosphatidylinositol (PI) (23). By this assay strain AG84 appeared to be specifically defective in sphingolipid synthesis.

It became necessary during the course of our experiments to transform AG84 with plasmid DNA. However, the strain transformed poorly, less than 10 transformants/μg of plasmid DNA.

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**TABLE I**

Genotypes and origin of yeast strains used in these studies

| Name      | Genotype                           | Relevant phenotype or gene | Source          |
|-----------|------------------------------------|-----------------------------|-----------------|
| YPH274    | MATα/α ura3–52/52 lys2–801mber ade2–101hvr trp1Δ/trp1Δ his3–Δ200/200 leu2–Δ1/leu2–Δ1 | Wild type, Lcb−           | Ref. 15         |
| RCD100    | MATα ura3–52/52 lys2–801mber ade2–101hvr trp1Δ/trp1Δ his3–Δ200/200 leu2–Δ1/leu2–Δ1 | Derivative of YPH274       | This work       |
| RCD101    | MATα ura3–52/52 lys2–801mber ade2–101hvr trp1Δ/trp1Δ his3–Δ200/200 leu2–Δ1/leu2–Δ1 | Derivative of YPH274, lcb3 deleted | This work       |
| RCD102    | MATα ura3–52/52 lys2–801mber ade2–101hvr trp1Δ/trp1Δ his3–Δ200/200 leu2–Δ1/leu2–Δ1 | Derivative of YPH274, YKR053C and lcb3 deleted | This work       |
| RCD103    | MATα ura3–52/52 lys2–801mber ade2–101hvr trp1Δ/trp1Δ his3–Δ200/200 leu2–Δ1/leu2–Δ1 | Derivative of YPH274, YKR053C and lcb3 deleted | This work       |
| SJ21R     | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Parent of 4R3, Lcb−        | Ref. 31         |
| 1A4       | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Parent of SJ21R, Lcb−      | Ref. 10         |
| 7R6       | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Parent of 114, Lcb−        | Ref. 10         |
| AG84–3    | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Parent of 7R6 carrying leu3–1 | This work       |
| LC3–1     | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Derivatives of AG84–3 carrying wild type | This work       |
| AGW–1, -2, -3, -4, -5, -6 | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Derivatives of AG84–3 carrying wild type | This work       |
| LC3–1     | MATα ura3–52/52 leu2–3, 112 ade1 MEL1 leu2–Δ1/leu2–Δ1 | Derivatives of AG84–3 carrying wild type | This work       |

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*Determination of Minimum Inhibitory Concentration*

Cells were grown overnight in minimal medium at 30 °C and diluted with fresh medium so that following addition of an inhibitory compound the final concentration was 1–2 × 10–5 ml, and the culture volume was 200 μl/microtitre well. Inhibitory compounds were tested in 2-fold dilution series in covered microtitre plates incubated without shaking at 30 °C for 40 h. The minimum inhibitory concentration is the lowest concentration of inhibitor yielding no visible cell pellet.

**Sphingoid Long Chain Base Utilization**
Sphingolipid synthesis was measured in vivo in AG84-3 and 7R6 cells as described under “Experimental Procedures” using protocol 1 with a 1-h incubation. Total [3H]inositol counts incorporated into sphingolipids represent the summation of the radioactivity in IPC, MIPC, and M(IP)2C separated by chromatography on Whatman SG-81 paper. Strains designations are: ○, 7R6; ■, AG84-3 transformed with pRS315; ▲, AG84-3 transformed with pJAB15-1 (LCB3); and ▼, AG84-3 transformed with pJAB2 (YKR053C).

To obtain a derivative of AG84 with enhanced transformability we selected derivatives that could grow on PYED plates at 37 °C. The rationale for this approach was to isolate a more stress-tolerant strain that would resist killing during the transformation procedure. Several 37 °C-resistant derivatives were tested for transformability, and one, AG84-3, was used for all studies presented here.

**Strain AG84-3 Cannot Make Sphingolipids When Fed PHS—** Synthesis of inositol-containing sphingolipids in AG84-3 cells was measured using an in vivo radiolabeling procedure. Cells were cultured overnight, without PHS to prevent sphingolipid synthesis, but with [3H]inositol to label PI, a substrate for synthesis of IPC and M(IP)2C. Cells were separated from unincorporated radioisotope and incubated with or without PHS in a buffered solution containing glucose. The parental strain 7R6, but not the presumptive mutant strain AG84-3, should exhibit a PHS-dependent transfer of the radiolabel from PI to ceramide to yield [3H]IPC and to [3H]MIPC to yield [3H]M(IP)2C. Parental strain 7R6 behaved as expected because the amount of [3H]-labeled sphingolipids synthesized was linearly dependent upon the concentration of PHS in the reaction (Fig. 2). In contrast, AG84-3 cells made no detectable sphingolipids during a 1-h (Fig. 2) or a 3-h (data not shown) incubation in the presence of 2.5 μM PHS. At 25 μM PHS, AG84-3 cells made sphingolipids, but at a lower level than 7R6 cells at both the 1-h (Fig. 2) and the 3-h time point (data not shown). Both strains showed deacylation products near the origin derived from deacylation of PI, indicating that synthesis of PI was normal (data not shown). From these data we conclude that AG84-3 cells have a defect in sphingolipid synthesis, specifically, they are less effective in transferring inositol-P from PI to ceramide to form IPC and to MIPC to form M(IP)2C (see Fig. 1). This behavior could be due to a defect in uptake of PHS from the culture medium, in synthesis of ceramide, or in transfer of inositol-P from PI to ceramide and MIPC.

To differentiate between a defect in PHS uptake and a defect in the biosynthetic pathway, a wild type copy of LCB1, encoding a subunit of serine palmitoyltransferase (Fig. 1), was introduced into strain AG84-3 (deleted for lcb1) on a plasmid, and sphingolipid synthesis was measured. If AG84-3 is defective in the transport of PHS then the LCB1 gene should restore sphingolipid synthesis because the strain will be able to make endogenous PHS, whereas the gene will not restore sphingolipid synthesis if the strain is defective in a later step in the biosynthetic pathway. Cells were cultured with [3H]inositol but not with PHS, and the amount of radiolabeled sphingolipids was measured as a function of time. AG84-3 cells (lcb1) carrying the LCB1 gene on a plasmid were able to synthesize sphingolipids as well as the parental strain 7R6 (LCB1) (Fig. 3). We conclude from these data that strain AG84-3 has a defect either in the uptake of PHS from the culture medium or in the intracellular transport of PHS to the site of ceramide synthesis in the endoplasmic reticulum.

**Isolation of a Gene That Restores PHS Incorporation into Sphingolipids in AG84-3 Cells—** A gene enabling strain AG84-3 to grow under restrictive conditions, 37 °C in the presence of 25 μM PHS, was isolated from a genomic library and designated LCB3. The rate of PHS incorporation into sphingolipids was measured in AG84-3 cells transformed with a centromeric vector carrying the cloned LCB3 gene as well as the parental strain 7R6 (LCB1) (Fig. 3). We conclude from these data that the LCB3 gene complements the defect in sphingolipid synthesis in AG84-3 cells and restores the rate of PHS incorporation into sphingolipids to the wild type level seen in 7R6 cells.

**LCB3 is predicted to encode a protein of 409 amino acids which lacks similarity to any known protein. It does show 53% amino acid identity (Fig. 4) to another S. cerevisiae open reading frame, YKR053C, whose predicted amino acid sequence also shows no similarity to any known protein or sequence motif.**

By one method (24) there are eight predicted transmembrane helices in the Lcb3 protein, and four of these (indicated in Fig. 4) are also predicted to be membrane-associated by two other methods (25, 26). Based on these predictions, the Lcb3 protein is most likely a membrane-bound protein having at least four and perhaps as many as eight transmembrane helices. Both the Lcb3 protein and the protein predicted to be encoded by open reading frame YKR053C have a putative glycosylphosphatidylinositol cleavage/attachment site at their COOH terminus (Fig. 4).

At this point we wanted to verify that mutation of the lcb3
gene and not some other gene, whose defect could be compensated indirectly by the $\text{LCB3}$ gene, was responsible for the sphingolipid synthesis defect in strain AG84-3. Segregation of the putative $\text{lcb3}$ mutant gene by tetrad analysis was not possible because strain AG84-3 does not sporulate when mated to another strain mutated in $\text{lcb1}$ and carrying the $\text{SLC1-1}$ suppressor gene (a strain like parental strain $\text{TR6}$ but of the opposite mating type). Instead of tetrad analysis, two other approaches were used to verify that the reduced rate of PHS incorporation into sphingolipids is due to inactivation of the $\text{lcb3}$ gene in AG84-3 cells.

First, if the cloned $\text{LCB3}$ gene is allelic to the mutated gene blocking incorporation of PHS into sphingolipids in strain AG84-3, then deletion of the $\text{lcb3}$ locus in strain AG84-3 should not relieve the block. Three independent derivatives of strain AG84-3 carrying the $\text{lcb3}$ deletion allele were constructed and found to behave the same. Only data for one strain (RCD104) will be presented. Like strain AG84-3, it made only very small amounts of sphingolipids when fed [3H]sphinganine (Fig. 5, top panel). We conclude from these experiments that an inactivating mutation in the $\text{lcb3}$ gene prevents exogenous long chain bases from being incorporated into sphingolipids at a normal rate in AG84-3 cells.

**Phenotypes of Wild Type Cells Carrying an $\text{lcb3}$ Deletion Allele**—All of the experiments described so far were performed in cells deleted for $\text{lcb1}$ and carrying the $\text{SLC1-1}$ suppressor gene (strains $\text{TR6}$, AG84-3, and their relatives). These strains grow slowly and die even under optimal conditions, and they could make sphingolipids. Direct measurement showed that incorporation of exogenous [3H]sphinganine into sphingolipids was restored in strain AGW-2 (Fig. 5, top panel). We conclude from these experiments that an inactivating mutation in the $\text{lcb3}$ gene prevents exogenous long chain bases from being incorporated into sphingolipids at a normal rate in AG84-3 cells.

![Figure 5: The $\text{LCB3}$ gene restores sphingolipid synthesis in strain AG84-3. Sphingolipid synthesis was measured by following the incorporation of exogenous [3H]sphinganine (0.91 μM) into sphingolipids for 4 h as described under "Experimental Procedures" using protocol 3. Sphingolipids were analyzed qualitatively by thin layer chromatography on Whatman LK5 plates with radioactivity localized using a BioScan apparatus as described in protocol 1. Brackets indicate the $\gamma$ axis scale in cpm. Peaks A, B, and C are M(IP)$_2$C species 3, 2, and 1, respectively (4); peak $D$ is an unknown; peak $E$ is MIPC-3; peaks $F$, $G$, and $H$ are IPC-3, 2, and 1, respectively; $O$ is the origin.](image5)

![Figure 4: Protein sequence alignment. The Lcb3 protein and the protein predicted to be encoded by open reading frame YKR053C were aligned (9) with identical amino acids indicated by a vertical bar and chemically similar amino acids by a dot. Predicted membrane-spanning domains (25, 26) are underlined, and predicted glycosylphosphatidylinositol cleavage/attachment sites (30) are indicated in bold.](image4)
RCD101) or the wild type \( LCB3 \) allele (strain RCD100) grew in PYED medium with the same doubling time at 30 °C, at pH 4.1, and in the presence of 0.75 M NaCl, indicating no effect of the \( lcb3 \) mutation by itself on the stress responses known to be defective in \( 7R6 \) cells when they lack sphingolipids (22).

If the Lcb3 protein transports long chain base into cells we would predict that the \( lcb3 \) deletion strain RCD101 should have a decreased rate of long chain base uptake. This prediction was examined by measuring incorporation of \( [3H] \) sphinganine into sphingolipids containing inositol-P. At a low concentration of sphinganine (0.91 \( \mu M \)) the \( lcb3-\Delta I \) mutant strain RCD101 made only low levels of sphingolipids compared with wild type RCD101 cells (Fig. 5, lower panel).

To quantify more carefully the effect of the \( lcb3-\Delta I \) deletion mutation, the time course of \( [3H] \) sphinganine incorporation into sphingolipids was measured. At a low concentration of sphinganine (0.65 \( \mu M \)), the \( lcb3 \) mutant strain showed almost no incorporation of radiolabel into sphingolipids over a 4-h time period, whereas the wild type strain showed a time-dependent increase in incorporation (Fig. 6). At a higher concentration of sphinganine (20.6 \( \mu M \)), the \( lcb3-\Delta I \) mutant strain showed a low level of incorporation which increased with time, but the level was less than half the wild type level (Fig. 6). We conclude from these data that the Lcb3 protein is necessary for a normal rate of incorporation of exogenous long chain base into sphingolipids.

If the Lcb3 protein transports sphingoid long chain bases into the cell, we would expect strains lacking the protein to be more resistant to growth inhibition by long chain bases and related hydrophobic compounds. As predicted, the \( lcb3-\Delta I \) deletion strain, RCD101, was more resistant to growth inhibition by several types of long chain bases, the water-soluble ceramide analog N-acetylsphingosine, and stearylamine than was the \( LCB3 \) strain, RCD100 (Table II). In addition, the deletion strain was more resistant to the toxic arginine analog canavanine and to the antibiotic hygromycin B. Both strains were toxified to the same degree by the serine palmitoyltransferase inhibitor (Fig. 1) sphingofungin B (23) and by the antibiotic cerulenin (Table II).

Because the Lcb3 protein is predicted to be homologous to the putative protein encoded by the \( YKR053C \) open reading frame, we determined if the two proteins were functionally related. A strain, RCD102, deleted for the \( YKR053C \) DNA sequence in a wild type genetic background, had the same level of resistance to long chain bases and other inhibitors of growth as the nondeleted strain, RCD100 (Table II). Likewise, the deleted strain had the same ability to incorporate exogenous \( [3H] \) sphinganine into sphingolipids as the \( lcb3 \)-deleted strain (Fig. 6). Based upon these data the protein encoded by \( YKR053C \) is not functionally related to the Lcb3 protein, at least not for the two phenotypes examined.

Based upon the reduced rate of \( [3H] \) sphinganine incorporation into the sphingolipids of \( lcb3 \)-defective cells over a 4-h period compared with wild type cells (Fig. 6), we anticipated that the initial rate of \( [3H] \) sphinganine uptake would also be reduced in the \( lcb3 \)-defective cells compared with wild type. Various conditions for measuring the initial rate of uptake were examined, but none proved satisfactory. For example, uptake during the first 3 min in wild type \( LCB3 \) (RCD100) and \( lcb3 \)-deleted cells (RCD101) was masked by what appeared to be rapid binding or adsorption of \( [3H] \) sphinganine to the cell surface, since wild type cells heated to 65 °C for 15 min bound at least half as many counts as did unheated cells (data not shown). In addition, no reproducible difference in uptake between nonheated wild type and \( lcb3 \) mutant cells could be detected.

**DISCUSSION**

Based upon the available data we conclude that the \( LCB3 \) gene encodes a membrane-bound protein necessary for utilization of exogenous long chain bases. The Lcb3 protein is predicted to be localized in the plasma membrane where we hypothesize that it transports or facilitates uptake of long chain bases. Alternatively, it could be bound to an internal membrane where it would promote delivery of long chain bases to the site of ceramide synthesis in the endoplasmic reticulum. \( LCB3 \) is the first gene in any organism to be implicated in long chain base transport.

Our conclusions and hypotheses are based upon an analysis of strain AG84-3, which is unable to make sphingolipids when fed PHS (Fig. 2). Failure to use exogenous PHS is not due to a block in one of the later steps in the sphingolipid biosynthetic pathway (Fig. 1) because the pathway is functional; complementation of the \( lcb1 \) mutation in AG84-3 cells with the \( LCB1 \) gene restores sphingolipid synthesis (Fig. 3). In addition, there was no difference in ceramide synthase (Fig. 1) activity between wild type RCD100 cells and cells deleted for either the \( lcb3 \) gene or the \( ykr053c \) open reading frame (data not shown).

We identified the mutant gene in AG84-3 cells, termed \( LCB3 \), by complementing the growth defect with a plasmid selected from a genomic DNA library. The Lcb3 protein is predicted to have four transmembrane domains by two algorithms (Fig. 4) and up to eight transmembrane domains by another algorithm (24) and is, therefore, most likely a membrane-bound protein. Another algorithm predicts that the protein is probably located in the plasma membrane (27). These predictions are consistent with the idea that AG84-3 cells are defective in a protein necessary for uptake of PHS from the culture medium.

The role of the \( LCB3 \) gene in PHS utilization was examined...
in a wild type, non-SLC genetic background to avoid the possibility that an unidentified mutation in strain AG84-3 was reducing PHS incorporation into sphingolipids. Deletion of the lcb3 gene in a wild type genetic background reduced the rate of \(^{3}H\)sphinganine incorporation into sphingolipids so that almost no incorporation was observed at a low substrate concentration (0.65 \(\mu\)g, Fig. 6) whereas some incorporation occurred at a higher concentration (20.6 \(\mu\)g, Fig. 6). These results support the hypothesis that the Lcb3 protein transports or facilitates uptake of long chain bases into cells. The finding that the lcb3-deleted strain RCD101 is more resistant than the nondeleted strain RCD100 to growth inhibition by long chain bases (Table II) further supports this hypothesis.

Attempts to measure directly the initial rate of long chain base uptake were not successful for at least two reasons. First, in the wild type genetic background even heat-killed cells contained a large amount of radioactive long chain base (sphinganine) after a 3-min incubation (data not shown), suggesting rapid binding to the cell surface, perhaps to the cell wall. The pmol of sphinganine bound/A\(_{600}\) of heat-killed cells by 3 min approaches the total long chain base content of cellular sphingolipids (28) and thus masks initial PHS uptake. Second, in a SLC genetic background the rapid putative surface binding was absent both in cells containing or lacking sphingolipids. Instead, there was a gradual increase over at least a 30-min period in the pmol of sphinganine bound/cell, both in heat-killed and nonkilled cells (data not shown). Because of these problems it was not possible to measure the initial rate of long chain base uptake.

Since wild type \(S.\ cerve\)vise\(a\) cells can make sphingoid long chain bases there would appear to be no need for the Lcb3 protein. However, it may normally perform an unidentified physiological function that is not necessarily under the growth conditions we examined. Alternatively, it may transport other compounds necessary for growth or survival under special, nonlaboratory circumstances. Cells lacking the lcb3 gene were more resistant to growth inhibition by the water-soluble, uncharged ceramide, N-acetylsphingosine, by the antibiotic hygromycin B, and by the arginine analog canavanine (Table II), indicating that the Lcb3 protein can recognize a wide range of compounds differing in charge, size, and hydrophobicity. Cells lacking the lcb3 gene were not more resistant to the serine palmitoyltransferase inhibitor sphingofungin B, an analog of sphingoid long chain bases (23), nor to the antibiotic cerulenin (Table II), suggesting that the Lcb3 protein does not transport these compounds into cells.

A search of the NCBI data bases revealed that the predicted Lcb3 protein was homologous to the protein predicted to be encoded by the \(S.\ cerve\)vise\(a\) open reading frame YKR053C but not to any other predicted proteins. The amino acid identity and similarity between these two proteins extends throughout their sequence, and both are predicted to have several membrane-spanning domains and a glycosylphosphatidylinositol cleavage/attachment site at their COOH terminus (Fig. 4). Despite their similarity in primary structure, they appear not to be functionally similar because a ykr053c deletion mutant did not show reduced incorporation of \(^{3}H\)sphinganine into sphingolipids like the lcb3 deletion mutant (Fig. 6), nor did the ykr053c deletion increase resistance to growth inhibition as did the lcb3 deletion (Table II). Based upon its primary sequence similarity to the Lcb3 protein, the protein encoded by YKR053C is likely to be a plasma membrane-bound protein. Analysis of a mutant strain deleted for both the lcb3 gene and the YKR053C open reading frame indicates that there is no synergy between the two genes because the double mutant and the lcb3 single mutant show the same reduction in incorporation of \(^{3}H\)sphinganine into sphingolipids (Fig. 6) and the same level of resistance to growth inhibition by long chain bases and other compounds (Table II).

In summary, a gene, LCB3, has been shown to be necessary for a normal rate of incorporation of exogenous long chain bases into sphingolipids. The Lcb3 protein is predicted to be located in the plasma membrane where it most likely transports long chain bases and other compounds into the cell. However, we cannot exclude the possibility of the Lcb3 protein being bound to an internal membrane such as the endoplasmic reticulum where it delivers long chain bases to the site of ceramide synthesis. The Lcb3 protein appears to represent a new class of membrane-bound protein because it shows no homology to any protein with a known function.

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