Sphingolipid long chain bases (LCBs) and phosphorylated derivatives, particularly sphingosine 1-phosphate, are putative signaling molecules. To help elucidate the physiological roles of LCB phosphates, we identified two Saccharomyces cerevisiae genes, LCB4 (YOR171c) and LCB5 (YLR260w), which encode LCB kinase activity. This conclusion is based upon the synthesis of LCB kinase activity in Escherichia coli expressing either LCB gene. LCB4 encodes most (97%) Saccharomyces LCB kinase activity, with the remainder requiring LCB5. Log phase lcb4-deleted yeast cells make no LCB phosphates, showing that the Lcb4 kinase synthesizes all detectable LCB phosphates under these growth conditions. The Lcb4 and Lcb5 proteins are paralogs with 53% amino acid identity but are not related to any known protein, thus revealing a new class of lipid kinase. Two-thirds of the Lcb4 and one-third of the Lcb5 kinase activity are in the membrane fraction of yeast cells, a puzzling finding in that neither protein contains a membrane-localization signal. Both enzymes can use phytosphingosine, dihydrosphingosine, or sphingosine as substrate. LCB4 and LCB5 should be useful for probing the functions of LCB phosphates in S. cerevisiae. Potential mammalian cDNA homologs of the LCB kinase genes may prove useful in helping to understand the function of sphingosine 1-phosphate in mammals.

The sphingolipid metabolite sphingosine and its phosphorylated derivative, sphingosine 1-phosphate (SPP), are thought to be signaling molecules for regulating a variety of mammalian cellular processes including cell growth, motility, and death (for review, see Refs. 1–3). Recently, SPP and sphingosine phosphorylcholine have been found to bind G protein-coupled receptors (for review, see Ref. 3) that may play roles in sylphosphorylation, death (for review, see Refs. 1–3). Recently, SPP and sphingosine kinases, referred to as LCB (long chain base) kinases, because they phosphorylate several LCBs including sphingosine. These two genes should expedite future studies of the functions of LCB phosphates in Saccharomyces and in multicellular eukaryotes.

MATERIALS AND METHODS

Strains, Plasmids, and Media—Strains used in these studies are listed in Table I. Strain MSS200 was made from strain JK9-3d by integrating the TP52-LacZ reporter plasmid pTP52-1 into the ura3 locus as described previously (10). Strain MSS206 is a derivative of MSS200 made by deleting DPL1 with the D allele followed by insertion of TRP1 into LEU2 by a marker swapping technique (11). The dpl1-D allele has the region between the Nhel and Bgl II restriction sites, codons 10–536, replaced by LEU2. Strain RCD158 is a derivative of JK9-3d MATa carrying lcb4-D1, a deletion allele with codons 1–560 replaced by a kanamycin resistance cassette. Strain RCD164 is a derivative of JK9-3d MATa carrying lcb5-D1, which has codons 3–683 replaced with a kanamycin resistance cassette. The kanamycin deletion alleles were generated by using the PCR, pUG6 (12) as a template, and a primer with 45 bases homologous to the sequence upstream or downstream of the deleted codons. Cells transformed with the deletion alleles were selected for G418 resistance (13). The expected deletion event was verified by PCR analysis of chromosomal DNA. RCD165 carries both lcb4-D1 and lcb5-D2 and was obtained as a metiotic segregant of diploid strain RCD167. RCD167 was made by crossing RCD165 carrying pRS315 (LEU2, 14) with RCD158, which had TP52-LacZ reporter plasmid integrated into the ura3 chromosomal locus to give Ura+ cells.

Strain MSS206 (dpl5) was mutagenized by transformation with a LEU2-tagged, transposon-mutagenized library of yeast genes essentially as described (15, 16). Transposon-tagged chromosomal genes were retrieved by digesting pRSQ2 (URA3) with BanHI followed by transformation into yeast and selection for Ura+ cells. Yeast chromosomal DNA was isolated, digested with EcoRI, self-ligated using T4 DNA ligase, and transformed into Escherichia coli strain DH5α.

The LCB4 and LCB5 coding regions were expressed in E. coli under control of the Trc promoter. The LCB4 coding region was amplified by using the PCR and the upstream primer 5’-CGAGGATCCGAGTGGTT-GTGCAAGAAAAC-3’ and the downstream primer 5’-CAGGAAGCTTCGATAGATTGAAACCTTG-3’ (the underlined bases are in LCB4). The PCR product was cleaved with BanHI and HindIII and ligated to pTrcHisB (Invitrogen) cleaved with the same two enzymes. The protein produced by this construct should have two extra amino acids (DP) at its amino terminus after cleavage with enterokinase. The LCB5 coding region was amplified in a similar manner by using the upstream primer 5’-CGAGGATCCGAGTGGTTGAAACCTTG-3’ and the downstream primer 5’-CAGGAAGCTTCGATAGATTGAAACCTTG-3’ (the underlined bases are in LCB5). The PCR product was cleaved with XhoI and HindIII and ligated to pTrcHisB cleaved with the same two enzymes. The protein produced by this construct should have five extra amino acids (DPSSS) at its amino terminus after cleavage with enterokinase. Plasmids were transformed into E. coli strain DH5α, gene expression
was induced by the addition of 1 mm isopropyl β-D-thiogalactopyranoside to the culture medium, and after 5 h growth at 30 °C, the cells were disrupted by five cycles of freezing and thawing as described in the Invitrogen manual. Disrupted cells were centrifuged for 5 min in a microcentrifuge, and the supernatant fluid was assayed for LCB kinase activity.

The composition of PYED (17) and defined media (18) used for growing yeast cells has been described. E. coli was grown in LB broth. Sphingosine (N-erythro-sphingosine, Matreya, Pleasant Gap, PA) was added to media in the presence of 0.05% tergitol (Nonidet P-40).

Preparation of Yeast Extracts—Yeast extracts were prepared by vortexing (6 × 30 s in a 15 ml Corex tube) 5–75 A 260 units of yeast cells in 1 ml of the extraction buffer (50 mM Hepes, pH 7.5; 5 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; and 1 μg/ml each of leupeptin, pepstatin, and aprotinin) with 0.5 ml of 0.5-mm-diameter acid-washed glass beads. All steps were done at 4 °C. The lysate was centrifuged for 20 min at 1,000 × g in a Sorvall SS-34 rotor. The resulting supernatant fluid was centrifuged at 100,000 × g for 15 min in a TLA 100.3 rotor (Beckman). The final supernatant fluid was frozen and stored at −20 °C. The final pellet was washed twice with extraction buffer, resuspended in 400 μl of buffer containing 20% glycerol, and frozen at −20 °C.

Assay of LCB Kinase Activity—The LCB kinase assay was based on the method of Crowther and Lynch (19). Reactions contained 7 μM DL-[3H]DHS ([20], 30 cpm/pmol, 20,000 cpm total), 0.5 mM Triton X-100, 1 mM MgCl2, 1 mM ATP, 100 mM Tricine, pH 8.1, and 1–50 μg of yeast proteins in a final volume of 100 μl. The reaction mixture was prepared by drying DHS under a stream of nitrogen, adding Triton X-100, and vortexing, followed by the addition of the other components. Enzyme was added to initiate the reaction; after incubation at 30 °C for 30 min, the product was separated from the substrate by differential solvent extraction (19). The amount of product formed was determined by liquid scintillation counting in Ultima Gold LSC-mixture (Packard). The Bradford reagent (Bio-Rad Laboratories) was used to measure protein concentrations with bovine serum albumin as a standard.

Miscellaneous Procedures—N-erythro-Dihydrosphingosine, C2-eremamide (N-acetylsphingosine), C3-eremamide, C5-erythro-dihydrosphingosine, and C5-erythro-sphingosine were purchased from Matreya. LCB phosphates were measured as [3P]-labeled compounds by growing cells overnight in PYED medium containing [3P]-labeled, and lacking exogenous potassium phosphate.

RESULTS

Optimization of Assay Conditions for S. cerevisiae LCB Kinase Activity—The assay conditions for LCB kinase activity have not been studied systematically in S. cerevisiae cells. We felt it was necessary to optimize the assay conditions as much as possible using cell extracts before isolation of mutants lacking enzyme activity. The components necessary for LCB kinase enzyme activity were determined using [3H]DHS as the LCB substrate. We verified that this substrate was converted to [3H]DHS-1-P by comparison of its mobility on a TLC plate with an authentic standard (data not shown).

As expected from previous studies of sphingosine kinase from mammals and other organisms (19, 21), the S. cerevisiae LCB kinase requires ATP and a detergent, Triton X-100, for activity (Fig. 1A). In the absence of detergent only about 10% of the [3H]DHS was solubilized in the reaction mixture. Complete Mg2+ dependence is evident from the partial loss of enzyme activity in the absence of exogenous Mg2+ and the complete inhibition of enzyme activity by EDTA in the absence of exogenous Mg2+. The reaction was not stimulated by 10 mM Ca2+ (data not shown). The reaction is linear with time for at least 60 min and with protein concentration up to about 25 μg (data not shown).

The reaction is linear with DHS concentrations up to about 5 μM; higher concentrations start to inhibit, and at 50 μM the reaction only goes to about 70% of the maximum value found at 5 μM (data not shown). The apparent Km values for DHS under our reactions conditions is 7 ± 0.6 μM (Fig. 2A). The apparent Km for ATP is 25 ± 3.2 μM (Fig. 2B). As discussed below, the Lcb4 kinase represents 97% of the activity in wild type cells; thus, these Km values are for the Lcb4 kinase.

Isolation of a Mutant Strain Lacking LCB Kinase Activity—Our rationale for identifying a gene encoding LCB kinase activity was based upon the observation that cells deleted for the DPL1 gene are more sensitive to growth inhibition by treatment with sphingosine than are parental cells (22). DPL1 is necessary for the breakdown of SPP to phosphoethanolamine and hexadecenal and is thought to encode the SPP lyase active site in S. cerevisiae cells (22). It seemed likely that sensitivity to sphingosine was caused by the accumulation of SPP and that inactivation of LCB kinase activity by mutation of the cognate gene would reverse the sphingosine-sensitive phenotype of the dpl1 deletion strain and allow cells to grow in the presence of an inhibitory concentration of sphingosine.

To be able to identify the mutated LCB kinase gene, MSS206 (relevant genotype is dpl1::J1) cells were transformed with a library of genes disrupted by insertion of a LEU2-tagged transposon (15). Leu+ transformants were pooled and replated on defined medium lacking leucine and uracil and containing 10 μM sphingosine. About 1/5,000 transformants grew, which was about the frequency expected for mutation of a single LCB kinase gene. However, MSS206 cells transformed with a con-
trol plasmid (pRS315, 14) gave resistant cells at a frequency of 1/8,000. We do not know why the control cells give such a high frequency of sphingosine-resistant cells, but this result indicated that not every MSS206 cell that lacked LCB kinase activity would be due to insertion of the \( \text{LEU2} \)-tagged transposon into the kinase gene.

To identify cells in which the kinase phenotype was caused by the insertion of the \( \text{LEU2} \)-tagged transposon into the kinase gene, we first assayed cells for LCB kinase activity and then verified by Southern blot analysis that there was only a single transposon per strain (15). 15 strains meeting these criteria were then analyzed in more detail by Southern blotting using chromosomal DNA cleaved either with \( \text{EcoRI} \) or \( \text{ClaI} \). These enzymes cleave the tagged transposon once and should yield two DNA fragments that hybridize to a radiolabeled transposon DNA probe. The rationale for this approach was to look for a correlation between the lack of LCB kinase activity and the size of the two restriction fragments because the size would depend upon the chromosomal location of the transposon.

Four LCB kinase strains were found to give the same restriction fragments, suggesting that the transposon had disrupted the same kinase gene. The chromosomal locus containing the transposon was retrieved from two of the strains by using a rescue plasmid. DNA sequence analysis of the retrieved DNA indicated that the \( \text{LEU2} \)-tagged transposon had integrated in both strains into open reading frame \( \text{YOR171c} \), renamed \( \text{LCB4} \) (Fig. 3).

A search of predicted \( \text{S. cerevisiae} \) proteins using the BLAST algorithm (23) identified the protein encoded by open reading frame \( \text{YLR260w} \), renamed \( \text{LCB5} \), as a homolog of the Lcb4 protein. The Lcb4 and Lcb5 proteins have 53% amino acid identity and 12% similarity, but most of these residues are concentrated in two regions within the carboxyl two-thirds of the proteins, residues 213–439 and 479–624 of Lcb4p (Fig. 3).

**FIG. 2.** LCB kinase activity as a function of substrate concentration. Panel A, DHS-1-P production was measured as a function of the DHS concentration and is presented as a double reciprocal plot yielding an apparent \( K_m \) of 7.7 ± 0.6 \( \mu \text{M} \) for DHS. Panel B, DHS-1-P production was measured as a function of the ATP concentration and is presented as a double reciprocal plot yielding an apparent \( K_m \) of 25 ± 3.2 \( \mu \text{M} \) for ATP.

**FIG. 3.** The Lcb4 and Lcb5 proteins are homologs. The predicted \( \text{S. cerevisiae} \) Lcb4p, Lcb5p, and the putative \( \text{S. pombe} \) homolog (PID, 2370540, GenBank accession no. Z98762) were aligned using the CLUSTAL algorithm (32) with identical amino acids indicated by an asterisk (*) and chemically similar amino acids by a dot (.). Gaps inserted into the sequences are indicated by a dashed line (—.—).
LCB kinase activity as shown by the protein- and time-dependent formation of DHS-1P (Fig. 4). Control extracts made from cells carrying the vector had no detectable LCB kinase activity (Fig. 4). We conclude from these experiments that LCB4 and LCB5 encode LCB kinase activity.

**RCRD158 (Δlcb4) Cells Contain No Detectable LCB Phosphates**—To determine which of the LCB genes is responsible for synthesis of LCB phosphates we quantified the amount of these compounds in mutant and parental cells. Cells were grown overnight in the presence of 32P, at 25 °C to early log phase (A600nm = 0.3) and then shifted to 37 °C for 10 min. Lipids were extracted as described previously, deacylated, and enriched for LCB phosphates by chromatography on an AG4 column. The fractions containing partially purified LCB phosphates were analyzed by thin layer chromatography. Parental JK9-3da cells (Fig. 5, lane labeled WT) make both DHS-1-P and PHS-1-P under these experimental conditions. The Δlcb4 (RCRD158) and Δlcb4 Δlcb5 (RCRD165) cells contain what appears to be a very small amount of radiolabeled compounds that migrate like the two LCB phosphates. However, when each sample was chromatographed in a second dimension, the two radioactive spots did not migrate like either PHS-1-P or DHS-1-P (data not shown). Thus, neither strain makes any detectable LCB phosphates. The Δlcb5 (RCRD164) cells contain a normal level of PHS-1-P and DHS-1-P compared with the wild type JK9-3da cells (Fig. 5). We conclude from these data that the Lcb4 kinase is responsible for synthesis of LCB phosphates under the conditions of these experiments; the Lcb5 kinase is unable to make any detectable LCB phosphates under these experimental conditions.

**Substrate Specificity of the Lcb4 and Lcb5 Kinases**—To begin to understand the function of each LCB kinase, we examined their substrate specificity because they might have different substrate specificity. In these experiments the concentration of [3H]DHS as the substrate. Data are the means ± S.D. for three assays. Specific enzyme activity is defined as the amount of enzyme needed to synthesize 1 pmol of DHS-1P/min.

| Strain   | Location | Distribution of enzyme units | Specific activity (pmol DHS-1P/min/mg protein) |
|----------|----------|-----------------------------|-----------------------------------------------|
| JK9-3da  | Soluble  | 34                          | 250 ± 17                                      |
| (wild type) | Pellet   | 66                          | 4,500 ± 95                                    |
|          | Total    | 100                         | 814                                           |
| RCD158   | Soluble  | 66                          | 11 ± 0.8                                      |
| (Δlcb4)  | Pellet   | 34                          | 54 ± 9.2                                      |
|          | Total    | 100                         | 21                                            |
| RCD164   | Soluble  | 31                          | 250 ± 5.4                                     |
| (Δlcb5)  | Pellet   | 69                          | 3,700 ± 185                                   |
|          | Total    | 100                         | 820                                           |
| RCD165 (Δlcb4 Δlcb5) (soluble and pellet) | | | 0 |

**TABLE III**

Substrate specificity of LCB kinases

Lcb4 and Lcb5 kinase activity was assayed using the soluble fraction prepared from RCD164 cells (deleted for lcb5) and RCD158 cells (deleted for lcb4). The concentration of [3H]DHS was 3.6 μM for the Lcb4 assays and 3.3 μM for the Lcb5 assays. The specific activity of the Lcb4 and Lcb5 kinases in the absence of inhibitor was 197 and 2.9, respectively. The competitors were all used at 3 μM. Data are the means ± S.D. for three assays. Specific enzyme activity is defined as the amount of enzyme needed to synthesize 1 pmol of DHS-1P/min/mg of protein.

| Competitor | Lcb4 kinase | % | Lcb5 kinase | % |
|------------|-------------|---|-------------|---|
| None       | 100 ± 2     | 100 ± 5    |
| PHS        | 70 ± 1      | 63 ± 2     |
| Sphingosine| 59 ± 2      | 46 ± 3     |
| Three-DHS  | 82 ± 0.05   | 105 ± 3    |
| C2-ceramide| 101 ± 1     | 104 ± 6    |

from [α-32P]ATP and the LCBs. The nonbiological threo isomer of DHS was only slightly inhibitory, indicating that it is a poorer substrate for the enzyme. C2-ceramide (N-acetyllysphin-
gosine), a compound lacking a free amino group, most likely necessary for a good enzyme substrate, showed no inhibitory activity toward either enzyme. Finally, the extracts used for these experiments (Table III) were frozen and thawed several times and exhibit lower specific activities for both kinases than are observed with fresh extracts (Table II). We conclude from the data presented in Table III that the Lcb4 and Lcb5 kinases have similar substrate specificities, at least under these assay conditions. More precise comparisons of substrate specificities must await purification of each enzyme.

**DISCUSSION**

Our data demonstrate that the LCB4 and LCB5 genes of *S. cerevisiae* encode LCB kinase activity, the first time that genes encoding this type of enzyme have been identified in any organism. This conclusion is based upon the complete lack of LCB kinase enzyme activity in an *lcbl lcb5* double deletion strain (Table II), upon the production of LCB kinase activity in *E. coli* cells expressing either LCB4 or LCB5 but not in cells carrying only the vector (Fig. 4), and finally, the demonstration that a *lcbl* deletion mutant does not make any detectable DHS-1-P or PHS-1-P in vivo under conditions where the parental strain makes both compounds (Fig. 5).

Identification of the *LCB4* gene was based upon the observation that a strain lacking the *DPL1* gene, believed to encode LCB phosphate lyase activity, is more sensitive to and is growth-inhibited by lower concentrations of sphingosine than is the parental strain (22). In the presence of sphingosine the mutant cells accumulate SPP (22) which is believed to inhibit growth, although the mechanism is unclear (25). Our data support the idea that SPP is growth-inhibiting since mutation of *lcbl* resulted in both the loss of more than 95% of cellular LCB kinase activity, and it allowed growth in the presence of an inhibitory concentration of sphingosine. Because SPP appears to inhibit growth it may be that under some physiological circumstances DHS-1-P, PHS-1-P, or both are used by yeast cells to modulate growth.

The predicted Lcb4 and Lcb5 proteins are paralogs that show no similarity to known proteins. They thus represent a new class of lipid kinase. They appear to be related to proteins with unknown functions including one from *S. pombe* (Fig. 3) and ones encoded by mouse and human expressed sequence tags (e.g. GenBank AA107451, D31133, R74736, and N55929). The two most conserved portions of the LCB kinases are in the middle and the COOH terminus of the proteins (Fig. 4). The functions of these regions and the less conserved NH2 terminus remain to be determined.

*S. cerevisiae* cells contain a high level of LCB kinase activity in the 100,000 × g pellet fraction based upon a specific activity of 4,500 pmol/mg/min (Table II). This value is more than four times the value of 1,080 pmol/mg/min for LCB kinase activity in corn shoot microsomes (19) and 100 times greater than the value of 40 pmol/mg/min from bovine brain (26). About two-thirds of the Lcb4 and one-third of the Lcb5 enzyme activity are found in the pellet fraction of the cell (Table II). It remains to be determined if the proteins are retained within the lumen of an organelle or if they are bound to a membrane. Neither protein has a predicted transmembrane spanning domain nor an attachment site for a lipid anchor, so if the proteins are binding to a membrane, they must do so through protein-protein interaction or through a novel lipid anchor motif. In platelets, sphingosine kinase is primarily a soluble enzyme, whereas in rat brain and other tissues it is peripherally associated with membranes (21). About 80% of the activity in rat pheochromocytoma PC12 neuronal cells is soluble and the other 20% is in the particulate fraction (27), where it is presumed to be membrane-bound. It should now be possible to determine the mechanism and the circumstances under which Lcb4 and Lcb5 enzyme activity distribute between the membrane and soluble fractions. This information may help to elucidate why sphingosine kinase activity is in a similar fraction in mammalian cells. Other lipid kinases have been shown to bind to membranes in a regulated manner (28), and it will be important to determine if LCB kinases show similar behavior.

Most of the LCB kinase activity in JK9-3a cells is encoded by LCB4 (Δlcbl, strain RCD164, Table II), and this kinase synthesizes all of the DHS-1-P and PHS-1-P present in log phase cells grown in rich medium because neither of these compounds was found in cells making only the Lcb5 kinase (Δlcb5, strain RCD158, Fig. 5). Our data do not eliminate the possibility that LCB phosphates made by the Lcb5 kinase are very short lived and present at a very low concentration. The function of the Lcb5 kinase may be very specialized and depend upon its cellular location. Alternatively, the LCB5 gene may just be a remnant of the genome duplication event that occurred in *S. cerevisiae* (29). This possibility seems unlikely, however, because a nonessential gene would be expected to become mutated and no longer encode an active enzyme. Mice and humans also seem to have more than one species of LCB kinase based upon our analysis of homologs predicted to be encoded by expressed sequence tags. Retention of multiple species of LCB kinases over a long evolutionary time span suggests that they perform specialized functions.

All eukaryotes examined to date have LCB kinase activity, indicating that this enzyme provides survival value to cells. Besides its presumed role in generating signaling molecules, it is essential for catalyzing LCBs to yield phosphoethanolamine, which serves as a precursor for synthesis of phosphatidyethanolamine and phosphatidylcholine. The physiological importance of this metabolic conduit from sphingolipid to glycerocephospholipid metabolism has not been determined. The LCB4 and LCB5 genes may make it possible to examine the role of this metabolic pathway in *S. cerevisiae* cells. Cells lacking both genes grow normally in rich and synthetic medium (data not shown), indicating that LCB phosphates and flux through this metabolic pathway are not necessary for a normal rate of growth with glucose as the carbon source, at least not under short term laboratory conditions. Other phenotypes await analysis as does the role of LCB phosphates as second messengers during stress responses (30).

If the putative human and mouse expressed sequence tags do indeed encode homologs of the yeast LCB kinases, it should be possible to use them to manipulate the level of SPP in mammals and thereby establish the physiological functions of SPP.

**Addendum—**After submission of this manuscript for publication, Olivera et al. (31) reported the purification and enzymatic characterization of sphingosine kinase from rat kidney.

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