

## Lcb4p Is a Key Regulator of Ceramide Synthesis from Exogenous Long Chain Sphingoid Base in *Saccharomyces cerevisiae*\*

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**Long chain sphingoid bases (LCBs) and their phosphates (LCBPs) are not only important intermediates in ceramide biosynthesis but also signaling molecules in the yeast, *Saccharomyces cerevisiae*. Their cellular levels, which control multiple cellular events in response to external and intrinsic signals, are tightly regulated by coordinated action of metabolic enzymes such as LCB kinase and LCBP phosphatase. However, little is known about the mechanisms by which the two enzymes generate biosynthetic or signaling outputs. It has been shown that the LCBP phosphatase, Lcb3p, is required for efficient ceramide synthesis from exogenous LCB. Here we present direct evidence that the major LCB kinase, Lcb4p, but not the minor kinase, Lcb5p, regulates synthesis of ceramide from exogenously added LCB. Surprisingly, our biochemical evidence suggests that the LCBP used for ceramide synthesis must be generated on the membrane. Our data show that Lcb4p is tightly associated with membranes and is localized to the endoplasmic reticulum where it can work in concert with Lcb3p. These results raise the conceptually attractive possibility that membrane-associated and cytosolic Lcb4p play distinct roles to differentially generate biosynthetic and signaling pools of LCBP.**

Sphingolipid metabolites, including ceramide, sphingosine, and sphingosine 1-phosphate (S1P)<sup>1</sup> function as important second messengers in mammalian cells, regulating diverse biological processes such as cell growth, differentiation, apoptosis, stress responses, calcium homeostasis, and cell migration (1–4). Several lines of evidence strongly suggest that the dynamic balance between intracellular ceramide/sphingosine and S1P is an important factor that determines their cellular processes (3, 5). However, the mechanisms by which cells regulate

intracellular levels of these lipids as well as their localization and mechanisms of action are largely unknown.

The level of S1P is regulated by the metabolic enzymes responsible for its formation, which is catalyzed by sphingosine kinase (6, 7), and its degradation, which is catalyzed by an endoplasmic reticulum (ER)-bound S1P lyase (8, 9) and a specific phosphatase (10, 11). In mammalian cells, two sphingosine kinase isoforms have been cloned and characterized (6, 7). Although sphingosine kinase type 1 (SPHK1) and type 2 (SPHK2) have a high degree of homology, they have differential tissue expression, temporal developmental expression, and properties, suggesting that they have distinct cellular functions and may regulate levels of S1P differently. Furthermore, SPHK1 is a cytoplasmic enzyme, whereas SPHK2 has several predicted transmembrane regions, suggesting that it is a membrane protein (3). However, both kinase activities are present in the cytosol and in membranes (6, 7). Another study suggested the presence of additional sphingosine kinases in mammalian tissues: one cytosolic and two membrane-bound activities that are associated with the ER and with plasma membrane (12).

In the yeast, *Saccharomyces cerevisiae*, two genes that encode related sphingosine kinases, named *LCB4* and *LCB5*, have been identified (13). They display 53% amino acid identity. The gene products, referred to as LCB (long chain sphingoid base) kinases, phosphorylate several LCBs, including dihydrosphingosine (DHS), phytosphingosine (PHS), and sphingosine. These predicted cytosolic kinases appear to have similar substrate specificities. Like mammalian kinases, two-thirds of the Lcb4p and one-third of the Lcb5p kinase activity are also found in the membrane fraction, although neither protein contains a membrane localization signal (13). Yeast also expresses a conserved S1P lyase encoded by the *DPL1* gene (14). Additionally, *LCB3* and *YSR3* encode LCB phosphate phosphatases (15–17). Both phosphatases are localized to the ER but appear to be functionally distinct (18).

Although the functions of LCB kinases in yeast are largely unknown, a study using yeast strains with deletions of LCB kinase genes showed that LCB kinases are important regulators of heat-induced cell cycle arrest, and that Lcb4p and Lcb5p function redundantly in this process (19). However, recent evidence suggests specific roles for the two LCB kinases. Lcb4p has a function in growth inhibition and cell death (20, 21) and Lcb5p plays a role in heat-stress resistance during induced thermotolerance (22). This difference implicates a complexity in the mechanisms in which the two LCB kinases control diverse cellular processes.

Earlier studies showed that deletion of *LCB3* leads to failure of incorporation of exogenous DHS into sphingolipids, suggesting a possible role for LCB phosphorylation and dephosphorylation in sphingolipid synthesis (16, 17). Here, we present evidence that incorporation of exogenous DHS into ceramide

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<sup>1</sup> The abbreviations used are: S1P, sphingosine 1-phosphate; AbA, aureobasidin A; DHS, dihydrosphingosine; DH-Cer, dihydroceramide; DHS-1P, dihydrosphingosine 1-phosphate; ER, endoplasmic reticulum; FuB, fumonisin B1; GPL, glycerolphospholipid; HA, hemagglutinin; IPC, inositolphosphorylceramide; LCB, long chain sphingoid base; LCBP, LCB phosphate; M(IP)<sub>2</sub>C, mannosyl-diinositolphosphorylceramide; PHS, phytosphingosine; SPHK1 and 2, sphingosine kinase types 1 and 2; Ab, antibody; mAb, monoclonal antibody; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DGPP, diacylglycerol pyrophosphate; C26, hexacosanoic acid.

TABLE I  
Yeast strains used in this study

Strain	Genotype
RH4402	<i>MAT<math>\alpha</math> lcb4<math>\Delta</math>::URA3 lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 leu2 bar1-1</i>
RH4423	<i>MAT<math>\alpha</math> pep4-3 ura3 leu2 bar1-1</i>
RH4427	<i>MAT<math>\alpha</math> lcb3<math>\Delta</math>::KanMx pep4-3 ura3 bar1-1</i>
RH4464	<i>MAT<math>\alpha</math> lcb3<math>\Delta</math>::KanMx lcb4<math>\Delta</math>::URA3 lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 bar1-1</i>
RH4835	<i>MAT<math>\alpha</math> ysr3<math>\Delta</math>::URA3 pep4-3 ura3 leu2 bar1-1</i>
RH4836	<i>MAT<math>\alpha</math> lcb3<math>\Delta</math>::KanMx ysr3<math>\Delta</math>::URA3 pep4-3 ura3 leu2 bar1-1</i>
RH4946	<i>MAT<math>\alpha</math> lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH4948	<i>MAT<math>\alpha</math> pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH4950	<i>MAT<math>\alpha</math> lcb4::LCB4 3xHA HIS5Sp lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH4951	<i>MAT<math>\alpha</math> lcb4<math>\Delta</math>::TRP1 lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH4953	<i>MAT<math>\alpha</math> lcb4<math>\Delta</math>::TRP1 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH4978	<i>MAT<math>\alpha</math> dpp1<math>\Delta</math>::HIS3 lpp1<math>\Delta</math>::TRP1 pep4-3 ura3 leu2 his3 trp1 lys2 bar1-1</i>
RH4981	<i>MAT<math>\alpha</math> dpp1<math>\Delta</math>::HIS3 lpp1<math>\Delta</math>::TRP1 lcb3<math>\Delta</math>::KanMx ysr3<math>\Delta</math>::URA3 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH5243	<i>MAT<math>\alpha</math> dpp1<math>\Delta</math>::HIS3 lpp1<math>\Delta</math>::TRP1 lcb4<math>\Delta</math>::URA3 lcb5<math>\Delta</math>::LEU2 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>
RH5244	<i>MAT<math>\alpha</math> dpp1<math>\Delta</math>::HIS3 lpp1<math>\Delta</math>::TRP1 pep4-3 ura3 leu2 his3 trp1 lys2 bar1-1</i>
RH5257	<i>MAT<math>\alpha</math> lcb5::LCB5 3xHA HIS5Sp lcb4<math>\Delta</math>::TRP1 pep4-3 ura3 leu2 his3 trp1 bar1-1</i>

requires both Lcb4p and Lcb3p. Using an *in vitro* ceramide synthesis assay, we showed a specific role for membrane-associated Lcb4p in ceramide synthesis. Although membranes lacking LCB kinases had normal LCBP phosphatase activity, they could not support ceramide synthesis from DHS-1P, suggesting that the actions of Lcb4p and Lcb3p must be concerted on the ER membrane. Our observations provide new insights into the mechanisms by which the cell might functionally separate biosynthetic and signaling pools of LCBP.

#### EXPERIMENTAL PROCEDURES

**Yeast Strains, Media, and Reagents**—The yeast strains used in this study are listed in Table I. For the deletion strains, the entire open reading frames were deleted and replaced with the designated genes. Deletions were confirmed by PCR. Three copies of the hemagglutinin (HA) epitope were introduced at the COOH terminus of Lcb4p and Lcb5p using a histidine selection as described previously (23). RH4950 and RH5257 strains were obtained from RH4946 and RH4953, respectively, by plating onto SD plates without histidine. Expression of Lcb3p tagged with FLAG peptide was performed with the same construct (pYSR2) as described previously (16). Strains containing pYSR2 were grown overnight in SD (synthetic minimal, 6.7 g/liter yeast nitrogen base without amino acids, 20 g/liter glucose, supplemented with the appropriate amino acids)-uracil (SD-ura) medium containing 2% glucose and resuspended in SD-ura medium containing 2% galactose. Expression of the tagged Lcb3p was then induced by incubating cells with SD-ura medium containing 2% galactose and detected by Western analysis using a mouse mAb M2 against FLAG (Sigma).

**In Vivo Labeling with [<sup>3</sup>H]DHS and [<sup>3</sup>H]DHS-1P**—Cell cultures, labeling of lipids with [<sup>3</sup>H]DHS (American Radiolabeled Chemical Inc., St Louis, MO), or [<sup>3</sup>H]DHS-1P prepared as below, lipid extraction, and treatment by mild alkaline hydrolysis with NaOH were performed as previously described (24). The labeled lipids were separated on thin-layer chromatography (TLC) plates (20 × 20, Merck, Darmstadt, Germany), which were developed in solvent system I (chloroform/methanol/4.2 N NH<sub>4</sub>OH = 9/7/2, v/v). Radiolabeled lipids were visualized and quantified on a Cyclone Storage phosphor system using a tritium-sensitive screen (Packard, Meriden, CT).

**Cytosol and Membrane Preparations**—Wild-type and mutant cytosol were prepared essentially as described previously (25). The preparation of total membranes was performed as described previously (26). In brief, spheroplasts (from 0.5 A<sub>600</sub> units of cells in log phase) were broken in lysis buffer (0.1 M sorbitol, 20 mM HEPES, pH 7.4, 150 mM potassium acetate, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml protease inhibitor mixture (pepstatin, leupeptin, and antipain)). Subsequently, unbroken cells and cell debris were removed by centrifugation at 3,000 × g for 10 min at 4 °C, and the resulting supernatants were centrifuged at 100,000 × g for 60 min at 4 °C to collect the membrane fraction. The pellet was washed twice and resuspended in B88 (20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol). Aliquots were frozen in liquid nitrogen and stored at -80 °C. Protein concentration was determined using the Bio-Rad protein assay kit.

**[<sup>3</sup>H]DHS-1P Preparation**—[<sup>3</sup>H]DHS-1P was synthesized enzymatically from [<sup>3</sup>H]DHS by incubation with wild-type yeast cytosol in the

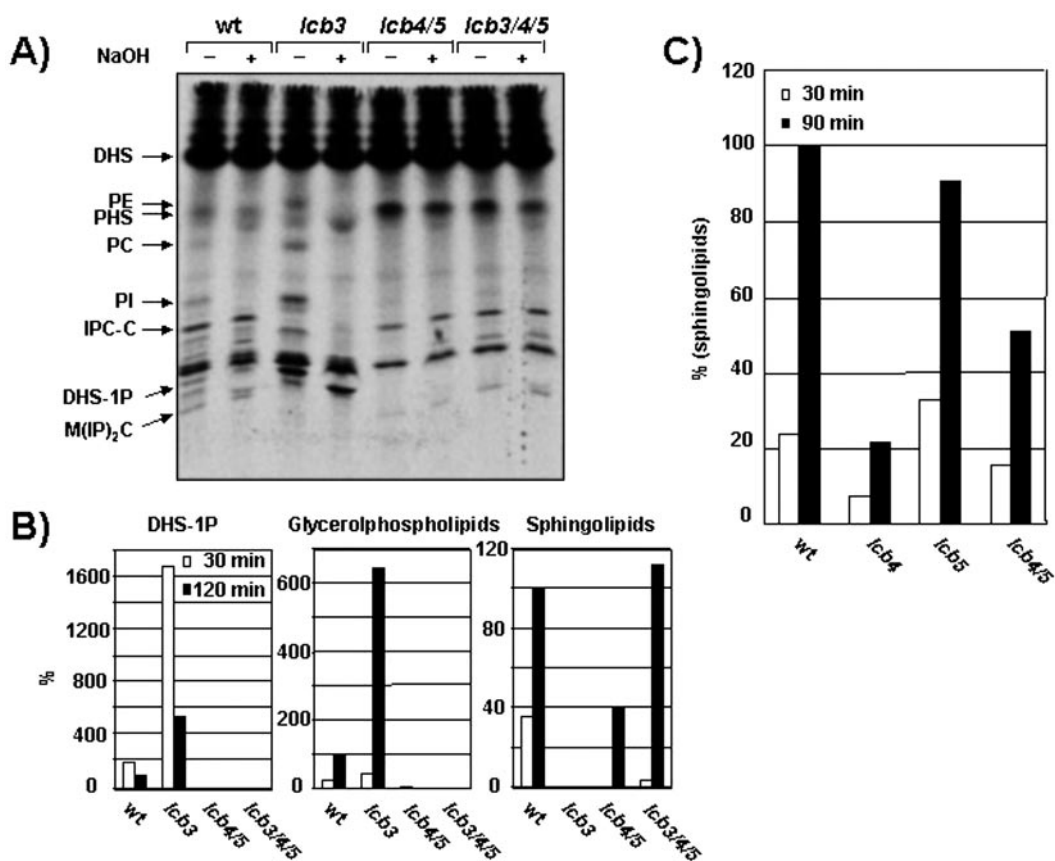
presence of an ATP-regenerating system (1 mM ATP, 40 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase). The synthesized [<sup>3</sup>H]DHS-1P was separated on TLC plates, which were developed in solvent system I, and then isolated from the TLC plates by scraping, and eluted with chloroform/methanol/water (10/10/3, v/v). The isolated [<sup>3</sup>H]DHS-1P was dried under nitrogen and partitioned between *n*-butanol and water as described (24). The butanol phase was dried under nitrogen, and lipids were dissolved in ethanol.

**In Vitro Labeling with [<sup>3</sup>H]DHS and [<sup>3</sup>H]DHS-1P**—*In vitro* ceramide synthase activity was determined as described previously (26). Membranes (200 μg), cytosol (100 μg), an ATP-regenerating system (1 mM ATP, 40 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase), GDP-mannose (50 μM), and either a mix of [<sup>3</sup>H]DHS and unlabeled DHS (10 and 40 pmol, respectively, 0.5 μCi) or [<sup>3</sup>H]DHS-1P (0.25 μCi) were first incubated for 15 min at 10 °C. Subsequently, CoA (50 μM) and a mix of liposomes containing hexacosanoic acid (C26) and phosphatidylinositol (PI) (50 μM/250 μM, respectively) were added, and the mixture was incubated for 2 h at 24 °C in a final total volume of 50 μl of B88. The reaction was stopped by addition of 333 μl of chloroform/methanol (1/1, v/v). The organic phase was collected after centrifugation at 13,000 × g for 5 min, and the pellet was re-extracted with 250 μl of chloroform/methanol/water. The extracted lipids were submitted to a mild alkaline treatment with NaOH and then partitioned between *n*-butanol and water and analyzed by TLC with solvent system II (chloroform/acetic acid = 9/1, v/v).

LCBP phosphatase activity was measured by adding [<sup>3</sup>H]DHS-1P (0.25 μCi) to the membranes (200 μg) in 50 μl of B88 containing apyrase (10 units/ml) and incubating at 24 °C for 60 min. The lipids treated with NaOH were analyzed by TLC with solvent system I as described above.

**Protein Extraction**—To characterize the nature of membrane association of proteins, cells were grown in SD-ura medium, washed, and resuspended with TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml protease inhibitor mixture). The cells were disrupted with glass beads, and the cell debris and glass beads were removed by centrifugation at 720 × g for 5 min at 4 °C. One volume of TNE buffer containing 2 M NaCl, 5 M urea, 2% Triton X-100, 2% SDS, or 200 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.5) was added to the supernatants (27). The mixture was incubated on ice for 30 min and centrifuged at 100,000 × g for 60 min at 4 °C. The resulting pellet and supernatant fractions were subjected to SDS-PAGE and then analyzed by Western blotting using a rat mAb 3F10 against HA (Roche Molecular Biochemicals, Basel, Switzerland), a mouse mAb M2 against FLAG, or a rabbit polyclonal antibody against End3p.

**Subcellular Fractionation**—For determination of the subcellular localization of proteins, spheroplasts were broken in lysis buffer as described above, and unbroken cells and cell debris were removed by centrifugation at 500 × g for 5 min at 4 °C. After centrifuging again at 500 × g for 5 min at 4 °C, the resulting supernatants (1 ml) were layered onto 1-ml steps of 22, 26, 30, 34, 38, 42, 46, 50, 54, and 60% (w/v) sucrose in 10 mM HEPES, pH 7.4, 1 mM MgCl<sub>2</sub> (28). The gradients were centrifuged at 200,000 × g for 140 min at 4 °C in a TST41.14 rotor. Eleven fractions of 1 ml were collected from the top of the gradient. Aliquots from each fraction were diluted with 10 mM HEPES, pH 7.4, 1 mM MgCl<sub>2</sub> and centrifuged at 100,000 × g for 60 min at 4 °C. Membrane pellets were then resuspended in B88. For analysis by immunoblotting, samples were solubilized in SDS sample buffer by vortexing and sub-



**FIG. 1. *LCB4* is required for an efficient incorporation of exogenous LCB into sphingolipids.** *A*, wild-type (RH4423), *lcb3* mutant (RH4427), *lcb4/5* mutant (RH4402), and *lcb3/4/5* mutant (RH4464) cells were labeled with [ $^3$ H]DHS for 2 h at 24 °C. The labeled lipids were subjected (+) or not (–) to mild alkaline hydrolysis with NaOH and applied to TLC plates using solvent system I. DHS (dihydrosphingosine), PE (phosphatidylethanolamine), PHS (phytosphingosine), PC (phosphatidylcholine), PI (phosphatidylinositol), IPC-C (inositolphosphorylceramide-C species), DHS-1P (dihydrosphingosine 1-phosphate), and M(IP) $_2$ C (mannosyl-diinositolphosphorylceramide) are marked. Unidentified lipids are not marked. *B*, cells were labeled with [ $^3$ H]DHS for 30 min or 2 h at 24 °C as above, and the labeled lipids were applied to TLC plates using solvent system I. Incorporation of [ $^3$ H]DHS into each lipid was quantified from the typical results obtained from three independent experiments. The relative amounts were expressed as percentages of the amounts in wild-type cells labeled for 2 h. GPLs include PE, PC, and PI; sphingolipids include IPC-C and M(IP) $_2$ C. *C*, wild-type (RH4948), *lcb4* mutant (RH4953), *lcb5* mutant (RH4946), and *lcb4/5* mutant (RH4951) cells were labeled with [ $^3$ H]DHS for 30 or 90 min at 24 °C. The incorporation of [ $^3$ H]DHS into sphingolipids was quantified, and the relative amounts were expressed as percentages of the amounts in wild-type cells labeled for 90 min as described in *B*.

jected to SDS-PAGE, followed by Western blotting using a rat mAb 3F10 against HA, a rabbit polyclonal Ab against Wbp1p or Emp47p, or a mouse mAb against the yeast vacuolar H $^+$ -ATPase 100-kDa subunit (Molecular Probes Europe BV, Leiden, Netherlands). The amount of protein in the fractions was quantified by densitometric scanning on a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

**Indirect Immunofluorescence Microscopy**—Indirect immunofluorescence on whole fixed yeast cells was performed as described by Beck *et al.* (29). For double labeling, a mouse mAb 12CA5 against HA (Roche Molecular Biochemicals, Basel, Switzerland) followed by an indocarbocyanine, Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and a rabbit polyclonal Ab against Kar2p (kindly provided by R. Schekman) followed by a fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used. Cells were visualized with a Zeiss Axiophot microscope (100 $\times$  objective).

## RESULTS

***LCB4* but Not *LCB5* Is Required for an Efficient Incorporation of Exogenous DHS into Sphingolipids**—*LCB3* and its homologue, *YSR3*, encode LCBP phosphatases in yeast (15). It has been demonstrated that *lcb3* deletion mutant cells fail to incorporate exogenously added DHS efficiently into ceramide and subsequently into sphingolipids, whereas endogenously synthesized LCBs are still converted to sphingolipids (16–18). These results suggest a possible role for a cycle of phosphorylation and dephosphorylation of exogenous LCB in ceramide synthesis. However, this has not been tested directly.

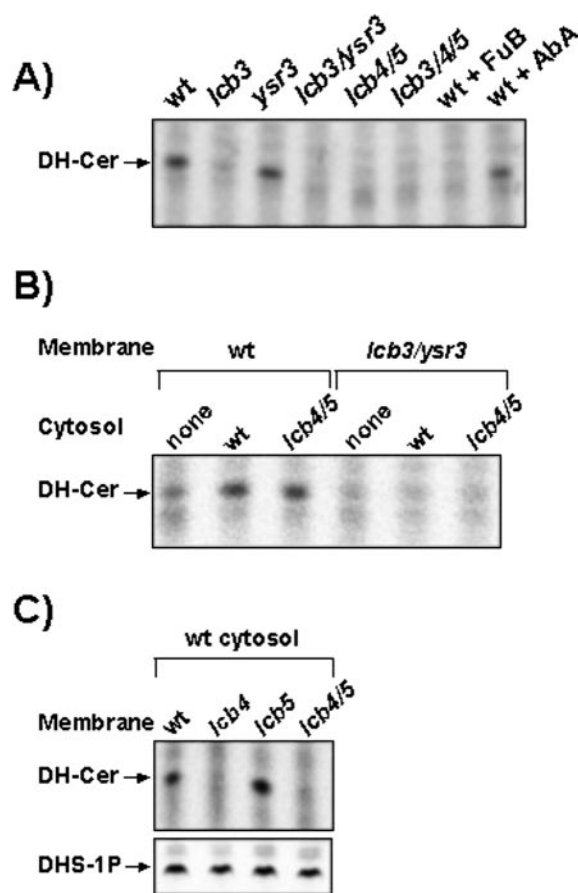
To test whether LCB kinase is required for incorporation of exogenous DHS into sphingolipids, we constructed multiple deletion strains for the two LCB kinases encoded by *LCB4* and *LCB5* (13) and measured sphingolipid synthesis by labeling with [ $^3$ H]DHS at 24 °C. The double *lcb4/5* deletion mutant cells could not synthesize DHS-1P (Fig. 1, *A* and *B*). This phenotype indicates that Lcb4p and Lcb5p are the only LCB kinases in yeast, as described previously (13). As expected, *lcb3* mutant cells accumulated DHS-1P. The *lcb3* mutant cells showed an 8-fold increase in DHS-1P synthesis after a short time labeling (labeling for 30 min) and a 5-fold increase after a long time labeling (labeling for 120 min) when compared with wild-type cells. Quantitative analysis also revealed that incorporation of [ $^3$ H]DHS into glycerolphospholipids (GPLs: PE, PC, and PI) in the *lcb3* mutant was increased 2- to 6-fold compared with wild-type cells throughout the 120-min labeling time. The decrease in DHS-1P in wild-type and *lcb3* mutant cells as GPL levels increase is consistent with a previous study, which suggested that DHS is incorporated into GPLs through phosphorylation of DHS and subsequent cleavage by the LCBP lyase, Dpl1p (16). This idea is further supported by our finding that the *lcb4/5* mutant, which fails to produce DHS-1P, could not synthesize GPLs from exogenously added DHS. Consequently, the *lcb4/5* mutant cells accumulated more PHS than *lcb3* mutant or wild-type cells (Fig. 1*A*).

Importantly, when the *lcb4/5* mutant cells were labeled with [<sup>3</sup>H]DHS for 30 min, the incorporation of [<sup>3</sup>H]DHS into sphingolipids (IPC-C and M(IP)<sub>2</sub>C) was hardly detectable (Fig. 1B) or was obviously reduced compared with wild-type cells (Fig. 1C). After longer labeling times, 120 min (Fig. 1B) and 90 min (Fig. 1C), the *lcb4/5* mutants showed 40–50% of the amount of sphingolipid synthesis found in wild-type cells. In contrast, when *lcb4/5* mutant cells were labeled with [<sup>3</sup>H]myo-inositol for 15 or 30 min at 24 °C, almost normal levels of all sphingolipids were observed (data not shown). This is consistent with a previous finding that *de novo* synthesis of sphingolipids is only slightly, if at all affected by deletion of both *LCB3* and *YSR3* genes (18). These results suggest that exogenous DHS must undergo a cycle of phosphorylation and dephosphorylation to be efficiently incorporated into sphingolipids.

We observed that the *lcb4* mutant cells could not make any detectable DHS-1P, as well as GPLs from exogenously added [<sup>3</sup>H]DHS, but the *lcb5* mutant cells could (data not shown). Therefore, we tested whether Lcb4p is the major kinase responsible for sphingolipid synthesis from exogenous DHS. To test this, we quantified the amount of synthesized sphingolipid in the single *lcb4* and *lcb5* mutant cells. The *lcb4* mutant cells showed 20–30% of the amount of sphingolipid synthesis found in wild-type cells, whereas the *lcb5* mutant cells showed only a slight reduction during a long time period of labeling (Fig. 1C). These results suggest that Lcb4p but not Lcb5p is required for an efficient sphingolipid synthesis from exogenous DHS.

During a long labeling period, *lcb4/5* mutant cells can synthesize detectable amounts of sphingolipids from exogenous DHS. In contrast, *lcb3* mutant cells synthesize no detectable sphingolipid during this time (Fig. 1, A and B). These results raised the question of whether Lcb3p is only required if exogenous DHS become phosphorylated. Indeed, a mutant lacking both the LCB kinases and the major phosphatase (*lcb3/4/5*) synthesized similar amounts of sphingolipids as wild-type cells in a long term labeling experiment (Fig. 1, A and B). After a shorter labeling time, the *lcb3/4/5* mutant resembled the *lcb3* or *lcb4/5* mutant in the incorporation of exogenous DHS into sphingolipids. Phenotypes in DHS-1P, GPLs, and PHS synthesis observed for the *lcb3/4/5* mutant are identical to the *lcb4/5* mutant. These results suggest that, if exogenous LCB does not get phosphorylated, it can bypass the normal route for incorporation into sphingolipid, which is dependent upon Lcb3p.

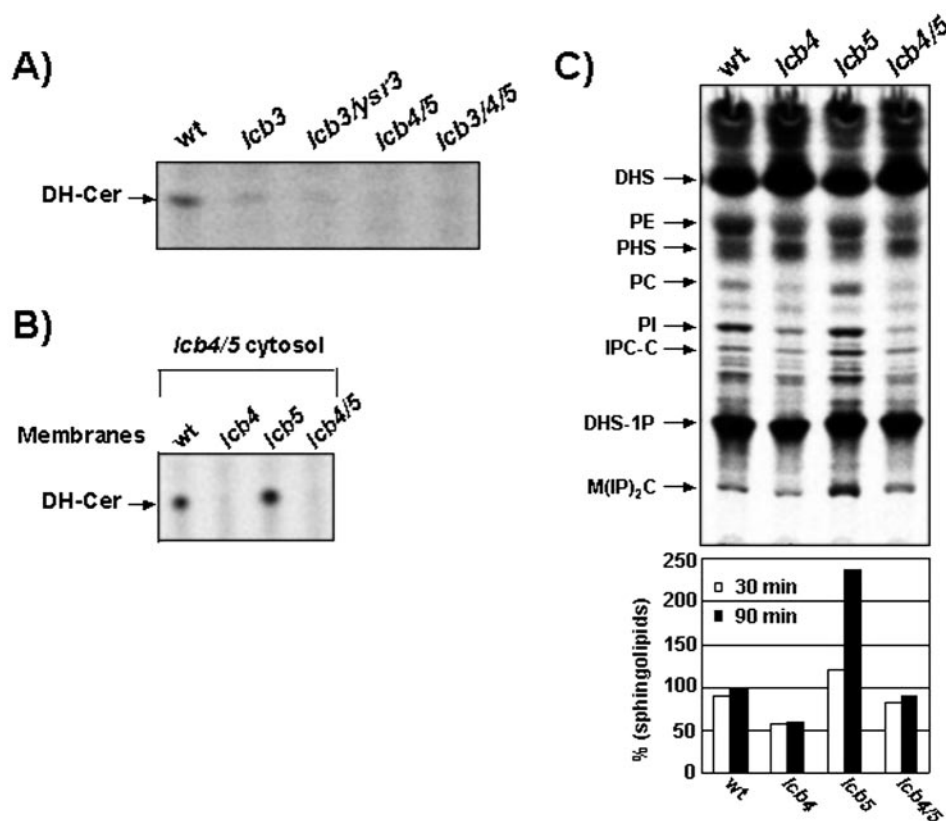
**Membrane-associated but Not Cytosolic Lcb4p Is Required for *In Vitro* Ceramide Synthesis**—The above data suggest that Lcb4p is required for an efficient ceramide synthesis from exogenous DHS. Therefore, we decided to measure ceramide synthase activity directly in an *in vitro* system that we recently developed (26, 30). *In vitro* ceramide synthase activity with [<sup>3</sup>H]DHS requires a membrane fraction, ATP, and cytosol (26). In addition, the reaction is temperature- and CoA-dependent (26, 30). When wild-type membranes were incubated with wild-type cytosol, an ATP-regenerating system, GDP-mannose, CoA, and a mix of liposomes containing C26 and PI, dihydroceramide (DH-Cer) was preferentially synthesized (Fig. 2A). Under the same conditions, very little phytoceramide was synthesized (Ref. 26 and data not shown). This DH-Cer synthesis was completely inhibited by fumonisin B1 (FuB), a specific inhibitor of ceramide synthase (31), but not by aureobasidin A (AbA), a specific inhibitor of IPC synthase (32). Membrane and cytosol derived from the double *lcb3/ysr3* mutant cells failed to synthesize DH-Cer. The single *lcb3* mutant showed a strong reduction of the incorporation of DHS into DH-Cer when compared with wild-type components, whereas *ysr3* mutant components showed normal synthesis (Fig. 2A). These results suggest that Lcb3p and Ysr3p do not have redundant functions in



**FIG. 2. LCB4 is essential for the FuB-sensitive *in vitro* ceramide synthesis from LCB.** A, membranes from wild-type (RH4423), *lcb3* mutant (RH4427), *ysr3* mutant (RH4835), *lcb3/ysr3* mutant (RH4836), *lcb4/5* mutant (RH4402), and *lcb3/4/5* mutant (RH4464) cells were labeled with [<sup>3</sup>H]DHS in the presence of cytosol prepared from the same strains. Fumonisin B1 (FuB) and aureobasidin (AbA) were added at 100  $\mu$ M and 50 nM, respectively. The labeled lipids, treated by mild alkaline hydrolysis, were applied to TLC plates using solvent system II. *In vitro* ceramide synthase activity was expressed as the amount of labeled DH-Cer (dihydroceramide). B, *in vitro* [<sup>3</sup>H]DHS labeling was performed with membranes from wild-type (RH4423) and *lcb3/ysr3* mutant (RH4836) cells in the absence or presence of cytosol from wild-type (RH4423) or *lcb4/5* mutant (RH4402) cells, and the labeled DH-Cer was analyzed as described in A. C, experiments were done under the same condition as in A, except that membranes from wild-type (RH4948), *lcb4* mutant (RH4953), *lcb5* mutant (RH4946), and *lcb4/5* mutant (RH4951) were labeled with [<sup>3</sup>H]DHS in the presence of wild-type (RH4423) cytosol and AbA (50 nM). The labeled lipids, DH-Cer and DHS-1P (dihydrosphingosine 1-phosphate) are noted.

ceramide synthesis. This is consistent with previous studies (18). Membrane and cytosol from either the *lcb4/5* mutant or the *lcb3/4/5* mutant had no detectable ceramide synthase activity. The complete lack of *in vitro* ceramide synthesis (Fig. 2A) and *in vitro* sphingolipid synthesis (data not shown) with *lcb3/ysr3*, *lcb4/5*, and *lcb3/4/5* mutants suggests that the *in vitro* assay measures only the efficient pathway for ceramide and sphingolipid synthesis that is mediated by LCB phosphorylation and dephosphorylation.

Because the incorporation of [<sup>3</sup>H]DHS into DH-Cer requires cytosol and membrane fractions (Ref. 26 and Fig. 2B), we next tested whether LCB kinase activity is required in the cytosol to make DH-Cer from [<sup>3</sup>H]DHS with wild-type membranes. Fig. 2B showed that an *lcb4/5* mutant cytosol fraction had an identical activity to support ceramide synthesis as wild-type cytosol, suggesting that cytosolic LCB kinases are not necessary for *in vitro* ceramide synthesis. This result also indicates that a cytosolic factor different from LCB kinases is required for *in*



**FIG. 3. Deletion of LCB4 affects *in vitro* ceramide synthesis as well as *in vivo* sphingolipid synthesis from exogenous LCBP.** *A*, membranes from wild-type and mutant cells indicated in Fig. 2*A* were incubated with [<sup>3</sup>H]DHS-1P in the presence of cytosol prepared from the same strains. The labeled lipid products, treated by mild alkaline hydrolysis, were applied to TLC plates using solvent system II. The labeled DH-Cer was quantified to determine the *in vitro* ceramide synthase activity. *B*, *in vitro* [<sup>3</sup>H]DHS-1P labeling was performed with membranes from wild-type and mutant cells indicated in Fig. 2*C*, in the presence of *lcb4/5* mutant (RH4402) cytosol and AbA (50 nM). The labeled DH-Cer was analyzed as described in *A*. *C*, *in vivo* [<sup>3</sup>H]DHS-1P labeling was done as described in Fig. 1*C*, except that [<sup>3</sup>H]DHS-1P was used instead of [<sup>3</sup>H]DHS. Incorporation of [<sup>3</sup>H]DHS-1P into sphingolipids was quantified, and the relative amounts were expressed as percentages of the amounts in wild-type cells labeled for 90 min.

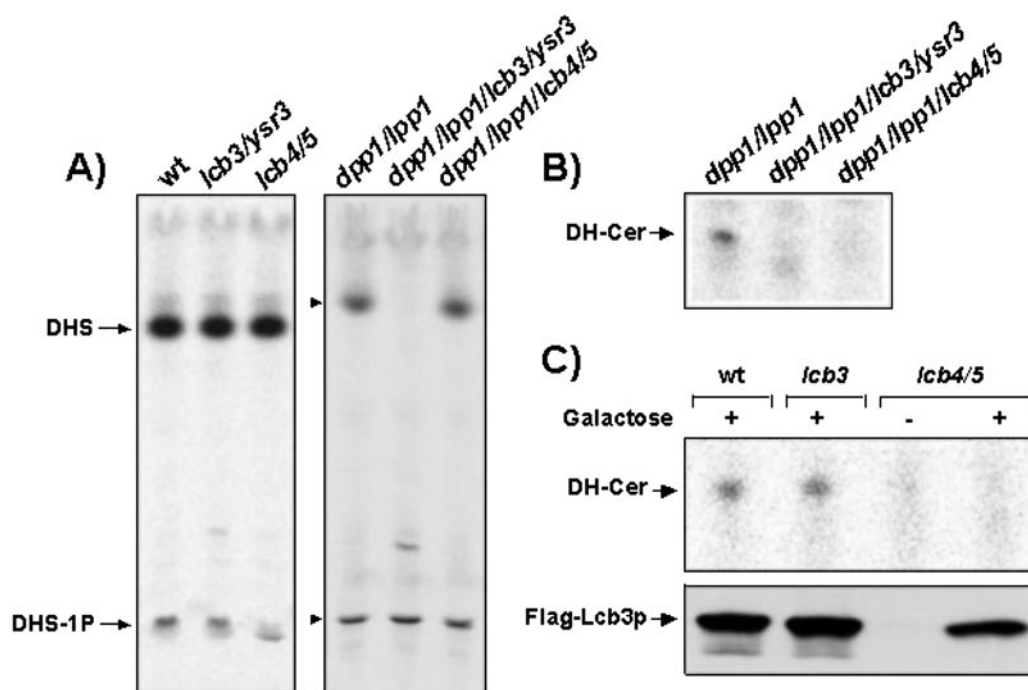
*in vitro* ceramide synthesis from [<sup>3</sup>H]DHS. On the other hand, membranes from the *lcb3/ysr3* mutant, mixed with either a wild-type or an *lcb4/5* mutant cytosol fraction, could not support ceramide synthesis. In addition, Fig. 2*C* shows that, when we measured ceramide synthase activity with the wild-type cytosol fraction, neither *lcb4* nor *lcb4/5* mutant membrane fractions had an activity to synthesize DH-Cer from [<sup>3</sup>H]DHS. In contrast, *lcb5* mutant membranes had an identical or an increased activity as compared with wild-type membranes, even though the amounts of DHS-1P produced in the reaction mixtures were similar. These exchange experiments suggest that *in vitro* ceramide synthesis requires membrane-associated but not cytosolic Lcb4p.

If DHS needs to be phosphorylated at the membrane to be converted into ceramide, the function of LCB kinase at the membrane should not be bypassed when we measure ceramide synthase activity with [<sup>3</sup>H]DHS-1P. Therefore, these mutant membranes were assayed for their ability to make DH-Cer directly from [<sup>3</sup>H]DHS-1P. Similar to the results observed with [<sup>3</sup>H]DHS (Fig. 2*A*), control experiments showed that the *lcb3* mutant and the *lcb3/ysr3* mutant, as well as *lcb3/4/5* mutant, almost completely blocked ceramide synthesis from [<sup>3</sup>H]DHS-1P (Fig. 3*A*). Our data showed that *in vitro* ceramide synthesis from [<sup>3</sup>H]DHS-1P was still defective in the *lcb4/5* mutant, suggesting that DHS-1P must be dephosphorylated and then rephosphorylated for incorporation into ceramide. Fig. 3*B* showed that, with the *lcb4/5* mutant cytosol fraction, the *lcb4* mutant membrane had no activity to synthesize DH-Cer from [<sup>3</sup>H]DHS-1P, whereas the *lcb5* mutant membrane had an iden-

tical or an increased activity as compared with the wild-type membrane. These results strongly suggest that the rephosphorylation is mediated by the membrane-associated but not cytosolic LCB kinase, Lcb4p. Thus, ceramide synthesis seems to be confined to the DHS-1P that is generated on the membrane.

We also measured *in vivo* sphingolipid synthesis from exogenous DHS-1P (Fig. 3*C*). When *lcb4* mutant cells were labeled with [<sup>3</sup>H]DHS-1P for 30 or 90 min, the incorporation of [<sup>3</sup>H]DHS-1P into sphingolipids was reduced to 50–60% of the wild-type amount. A lesser effect was observed for the *lcb4/5* mutant cells. In contrast, the *lcb5* mutant cells showed an increased incorporation of [<sup>3</sup>H]DHS-1P into sphingolipids compared with wild-type cells. A similar increase in incorporation into sphingolipids was seen when the *lcb5* mutant cells were labeled with [<sup>3</sup>H]DHS for 30 min (Fig. 1*C*). These results suggest that Lcb4p is required for an efficient sphingolipid synthesis from exogenous DHS-1P as well as DHS (Fig. 1*C*). This is consistent with our *in vitro* finding that the function of Lcb4p in ceramide synthesis was not bypassed when labeling with [<sup>3</sup>H]DHS-1P (Fig. 3*B*). In addition, we found that the *lcb4/5* mutant, as well as the *lcb4* mutant cells, could synthesize GPLs from [<sup>3</sup>H]DHS-1P and accumulate similar amounts of PHS as wild-type cells (Fig. 3*C*). These phenotypes are different from those observed with [<sup>3</sup>H]DHS (Fig. 1, *A* and *B*).

*The in Vitro Ceramide Synthesis Defect in the lcb4 Mutant Is Not Due to the Lack of LCBP Phosphatase Activity*—A defect in ceramide synthesis *in vitro* observed with the *lcb4* mutant membranes might result from the lack of LCBP phosphatase activity. Therefore, we measured phosphatase activity in mem-

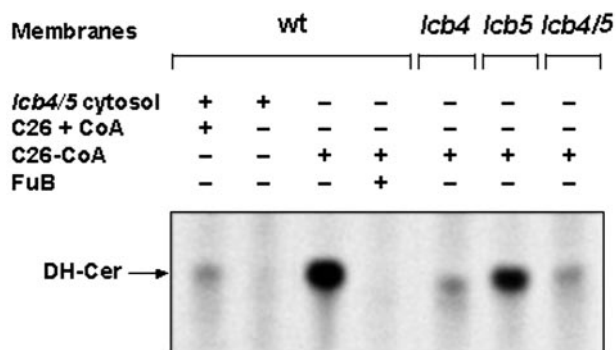


**FIG. 4. The *in vitro* ceramide synthesis defect in *lcb4/5* mutant membranes is not due to the lack of LCBP activity ascribed to Lcb3p/Ysr3p.** **A**, LCBP activity in membranes from wild-type (RH4423), *lcb3/ysr3* mutant (RH4836), *lcb4/5* mutant (RH4402), *dpp1/lpp1* (RH4978 or RH5244) mutant, *dpp1/lpp1/lcb3/ysr3* (RH4981) mutant, and *dpp1/lpp1/lcb4/5* (RH5243) mutant cells was measured by using [ $^3$ H]DHS-1P as the substrate, as described under "Experimental Procedures." **B**, membranes from *dpp1/lpp1* (RH4978 or RH5244) mutant, *dpp1/lpp1/lcb3/ysr3* (RH4981) mutant, and *dpp1/lpp1/lcb4/5* (RH5243) mutant cells were labeled with [ $^3$ H]DHS-1P in the presence of *lcb4/5* mutant (RH4402) cytosol and AbA (50 nM). The labeled DH-Cer was analyzed for *in vitro* ceramide synthase activity as described in Fig. 3A. **C**, wild-type (RH4423), *lcb3* mutant (RH4427), and *lcb4/5* mutant (RH4951) cells were transformed with plasmid pYSR2, carrying LCB3 tagged with FLAG, which was expressed under control of the Gal1 promoter. Cells were grown overnight at 24 °C in SD-ura medium containing glucose, resuspended in SD-ura medium containing galactose (+) or glucose (-), and incubated at 24 °C for 4 h. Membranes derived from these cells were labeled with [ $^3$ H]DHS-1P in the presence of *lcb4/5* mutant (RH4402) cytosol and AbA (50 nM). The labeled DH-Cer was analyzed as described above. The expression of FLAG-Lcb3p in membranes was analyzed by SDS-PAGE followed by Western blotting.

brane fractions by monitoring the production of DHS from [ $^3$ H]DHS-1P. Preliminary experiments, however, showed that *lcb3/ysr3* mutant membranes as well as *lcb4/5* mutant membranes had LCBP phosphatase activity similar to wild-type membranes (Fig. 4A), suggesting that there are other phosphatases that can utilize DHS-1P as substrates *in vitro*. Mg<sup>2+</sup>-independent phosphatidate phosphatase activity of yeast diacylglycerol pyrophosphate (DGPP) phosphatase has been shown to be inhibited by sphingosine 1-phosphate and ceramide 1-phosphate *in vitro* (33). These results suggest that these lipid phosphates are substrates for DGPP phosphatase. The *DPP1* gene and its homologue *LPP1* have both been shown to encode DGPP phosphatase activity (34, 35). Therefore, we combined *dpp1* and *lpp1* mutations with *lcb4/5* and *lcb3/ysr3* to construct LCB kinase and LCBP phosphatase mutants in a *dpp1/lpp1* background, respectively. Unlike *lcb3* or *lcb3/ysr3* mutant strains, the *dpp1/lpp1* strain showed normal sphingolipid synthesis when analyzed by *in vivo* labeling with [ $^3$ H]DHS as well as with [ $^3$ H]myo-inositol (data not shown). As expected, the membrane fraction prepared from the *dpp1/lpp1/lcb3/ysr3* mutant strain showed no detectable activity to dephosphorylate DHS-1P (Fig. 4A). Importantly, the *dpp1/lpp1/lcb4/lcb5* mutant membrane fraction had an identical activity when compared with the *dpp1/lpp1* mutant as control strain. These results suggest that *lcb4/5* mutant membranes contain normal Lcb3p and/or Ysr3p activity. In addition, *in vitro* ceramide synthase activity with [ $^3$ H]DHS-1P in the *dpp1/lpp1/lcb4/lcb5* mutant membranes was still not detectable (Fig. 4B). Consistent with these results, overexpression of FLAG-Lcb3p restored ceramide synthase activity in the *lcb3/ysr3* mutant but not in the *lcb4/lcb5* mutant membranes (Fig. 4C). Therefore, we conclude

that the *in vitro* ceramide synthesis defect in *lcb4* mutant membranes is not due to the lack of LCBP phosphatase activity.

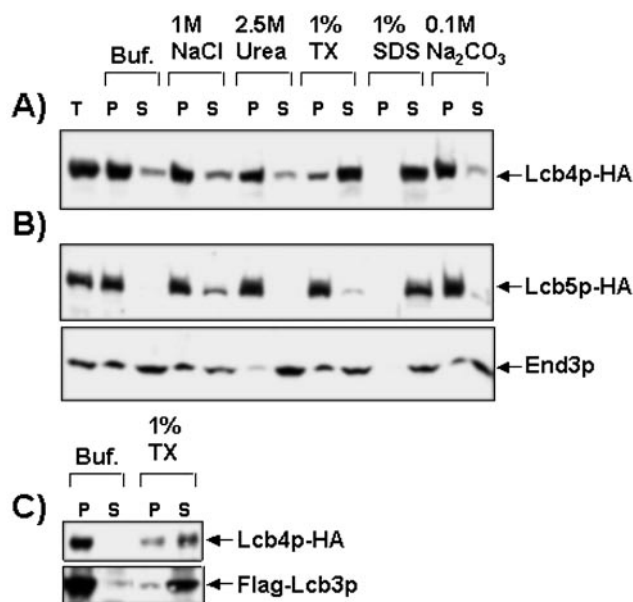
*Lcb4p Is Required for FuB-sensitive and C26-CoA-dependent in Vitro Ceramide Synthesis*—The above data suggest that membrane-associated Lcb4p is required for ceramide synthesis in our *in vitro* system, which is sensitive to FuB (Fig. 2A) and requires a cytosolic protein (Fig. 2B) and CoA (30). We also found that, when both CoA and C26 were omitted from the assay, the *in vitro* ceramide synthase activity with [ $^3$ H]DHS in wild-type membranes was strongly reduced (Fig. 5). Another *in vitro* system was developed to measure FuB-sensitive DH-Cer synthase activity with the use of C26-CoA as substrate (36), instead of free C26 and CoA. Activity in this system does not require cytosol. Therefore, we decided to measure *in vitro* ceramide synthase activity more directly by this *in vitro* system with C26-CoA (Fig. 5). When the assay was performed with wild-type membranes in the presence of a similar amount of C26-CoA (175  $\mu$ M) to free C26 and CoA (100 and 200  $\mu$ M, respectively) and in the absence of cytosol, the radiolabeled DH-Cer from [ $^3$ H]DHS increased 12-fold, as compared with the level seen in the presence of free C26, CoA, and *lcb4/5* cytosol. The increased C26-CoA-dependent ceramide synthesis was completely blocked by FuB, as described previously (36). When membranes from *lcb4* mutant or *lcb4/5* mutant cells were included in this assay, we observed only 9–10% of the DH-Cer synthase activity found in wild-type membranes. The small amount of DH-Cer production could be due to the presence of Zwittergent 3–16 (15  $\mu$ M in reaction mixtures), which was used to add C26-CoA, because the membranes could be partially solubilized by Zwittergent 3–16. On the other hand, membranes from the *lcb5* mutant showed about 50% of the wild-



**FIG. 5. LCB4 is required for C26-CoA-dependent *in vitro* ceramide synthesis from LCB.** Membranes from wild-type and mutant cells indicated in Fig. 2C were labeled with [ $^3$ H]DHS under standard conditions described under "Experimental Procedures," except that liposomes containing hexacosanoic acid (C26) were made with PC instead of PI. The experiments were done in the presence (+) or absence (-) of *lcb4/5* mutant cytosol (2 mg/ml), C26 and CoA (100 and 200  $\mu$ M), C26-CoA (175  $\mu$ M), or FuB (200  $\mu$ M). The labeled lipids, treated by mild alkaline hydrolysis, were applied to TLC plates using solvent system II. *In vitro* ceramide synthase activity was determined from the amount of labeled DH-Cer.

type DH-Cer synthase activity. These results suggest that Lcb4p does not act in the synthesis of C26-CoA but, rather, in the synthesis of LCBP and/or its delivery to the compartment where Lcb3p is localized.

**Lcb4p and Lcb5p Are Tightly Associated with Membranes in Distinct Manners**—LCB kinase activity has been found in both soluble and membrane fractions, although neither LCB kinase protein contains a membrane localization signal (13). By measuring the production of DHS-1P from [ $^3$ H]DHS under our *in vitro* conditions, we also found that LCB kinase activity is in both cytosolic and membrane fractions (data not shown). In addition, Lcb4p must be membrane-associated for ceramide synthase activity in our assay. Therefore, we next investigated the nature of the membrane association of the LCB kinases, Lcb4p and Lcb5p. We tagged the COOH terminus of the endogenous LCB genes with three copies of the HA epitope tag. Both Lcb4p-HA and Lcb5p-HA proteins are functional, because when expressed they complement the LCB phosphorylation defect of an *lcb4/lcb5* mutant (data not shown). Total cell lysates prepared from strains expressing Lcb4p-HA or Lcb5p-HA as the only LCB kinase, were treated with reagents that can extract proteins from membranes, and then centrifuged at 100,000  $\times g$  for 60 min. The presence of proteins in the supernatant (S) and pellet (P) fractions was determined by Western blotting. The majority of Lcb4p-HA was found in the membrane pellets (Fig. 6A). A similar result was observed with Lcb5p-HA (Fig. 6B). When the lysates were treated with 1% SDS, both Lcb4p-HA and Lcb5p-HA were completely released into the soluble fraction. However, although 1% Triton X-100 extracted Lcb4p-HA (Fig. 6A), Lcb5p-HA was not solubilized by 1% Triton X-100 (Fig. 6B), suggesting that Lcb5p-HA is associated with a detergent-insoluble structure. When cell lysates were treated with 1 M NaCl, 2.5 M urea, or 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.5), neither Lcb4p-HA nor Lcb5p-HA were extracted from membrane pellets by these treatments. As a control, we also checked End3p, a peripheral membrane protein that is required for the internalization step of endocytosis and has a role modifying the actin cytoskeleton (37). End3p was extracted almost completely by urea and partially by Na<sub>2</sub>CO<sub>3</sub> but was not solubilized by 1% Triton X-100 (Fig. 6B), as described previously (37). In contrast, the integral membrane protein Lcb3p was completely solubilized by 1% Triton X-100, as was Lcb4p (Fig. 6C). These results suggest that membrane association of both Lcb4p and



**FIG. 6. Both Lcb4p-HA and Lcb5p-HA are tightly associated with membranes with physically different natures.** A, cell lysates were prepared from Lcb4p-HA-expressing *lcb5* mutant (RH4950) cells as described under "Experimental Procedures," treated with various chemical reagents as indicated, and then centrifuged at 100,000  $\times g$  for 60 min. Total (T), soluble (S), and pellet (P) fractions were subjected to SDS-PAGE followed by Western blotting. B, experiments were done with Lcb5p-HA-expressing *lcb4* mutant (RH5257) cells as described in A. C, Lcb4p-HA-expressing *lcb5* mutant (RH4950) cells transformed with plasmid pYSR2 were grown in SD-ura medium containing galactose, as described in Fig. 4C. The cell lysates were treated with or without Triton X-100 and centrifuged at 100,000  $\times g$  for 60 min. Soluble (S) and pellet (P) fractions were subjected to SDS-PAGE followed by Western blotting.

Lcb5p is very strong but that the nature of the association is different for the two proteins.

**Lcb4p Co-fractionates with ER and Golgi, Whereas Lcb5p Co-fractionates with the Golgi**—Because Lcb4p and Lcb5p are tightly associated with membranes, we next investigated to which membrane they localize. The subcellular localization of Lcb4p was initially examined by fractionating membranes expressing Lcb4p-HA on a sucrose density gradient. Fig. 7A shows that the majority of Lcb4p-HA co-fractionated with the ER marker, a subunit of oligosaccharyltransferase, Wbp1p, even though some of the Lcb4p-HA was also found with Emp47p, a specific Golgi marker (28). Lcb4p-HA did not co-fractionate with the vacuole marker Vph1p (38). These results suggest that Lcb4p is localized primarily in the ER and Golgi. The ER association is consistent with location of Lcb3p (18), as well as subsequent enzymes in ceramide synthase, Lag1p and Lac1p (39). These results are also consistent with our previous finding that *in vitro* ceramide synthase activity is localized in the ER-enriched membranes (26). Interestingly, Lcb5p-HA co-fractionated tightly with Emp47p (Fig. 7B), suggesting that Lcb5p is localized in the Golgi. Therefore, we conclude that Lcb4p, but not Lcb5p, is present in the ER.

**Lcb4p Co-localizes with an ER Marker, Kar2p**—To confirm the ER localization of Lcb4p, we performed an indirect immunofluorescent microscopic analysis (Fig. 8). Visualization of Lcb4p-HA using monoclonal anti-HA antibodies revealed a discrete punctate staining, as well as a ring-like staining typical of ER-localized proteins in the interior of cells. Lcb4p-HA was also observed in the vacuole. The localization of Lcb4p-HA in the vacuole membrane is probably seen because of a vacuole protease defect due to the *pep4* mutation. Double staining with antibodies against Kar2p, an ER marker, showed that

Lcb4p-HA partially co-localized with Kar2p. Clearly, Kar2p and Lcb4p-HA were both found in the ER, but they labeled the ER in a somewhat distinct pattern. These results indicate that Lcb4p is localized to the ER and other organelles. Recently, Hait *et al.* (40) reported that Lcb4p-HA co-localizes with Sec7p, Tlg1p, or Pep12p, indicating localization in the Golgi and the

endosomes. Thus, the two immunofluorescence studies are in agreement with our biochemical fractionation studies (Fig. 7) and demonstrate that Lcb4p is partly associated with the ER and partly with other organelles. We were unsuccessful in localizing Lcb5p by immunofluorescence.

## DISCUSSION

In this study we have shown that exogenous LCB needs to undergo a cycle of phosphorylation and dephosphorylation to be efficiently incorporated into ceramides and sphingolipids by *S. cerevisiae* cells. This cycle can be bypassed, and sphingolipids are synthesized, albeit less efficiently, if exogenous LCB cannot be phosphorylated. Surprisingly, we found that the phosphorylation/dephosphorylation events must take place at the membrane for incorporation into ceramide via the efficient pathway. Lcb4p shows a tight membrane association and is localized to the ER, consistent with its function in converting exogenous LCB into ceramide. These results suggest that the intracellular localization of LCB phosphorylation is critical in determining its subsequent function in ceramide biosynthesis, phospholipid biosynthesis, or signaling.

A possible role for a cycle of phosphorylation and dephosphorylation of exogenous LCB in ceramide and sphingolipid synthesis has been suggested, because the deletion of *LCB3* almost completely blocks the incorporation of exogenous DHS into ceramide and sphingolipids (16–18). We showed that *lcb4* deletion mutant cells, as well as the *lcb4/5* deletion mutant, had a strong reduction in the incorporation of exogenous DHS into sphingolipids in short term labeling (Fig. 1). This observation is consistent with the previous studies, and thus, Lcb4p functions within an efficient pathway in sphingolipid synthesis from exogenous LCB. On the other hand, loss of function of LCB kinases had a significantly weaker effect on the process than deletion of the LCBP phosphatases in long-term labeling, suggesting the presence of an alternative pathway. The alternative pathway does not require a cycle of phosphorylation and dephosphorylation, because a triple mutant strain deleted for *LCB3*, *LCB4*, and *LCB5* genes is still able to make sphingolipids from exogenous DHS over a longer time. The mechanism of this alternative pathway remains unclear, but it seems to be more active in *lcb3* mutant cells, because the *lcb3/4/5* mutant incorporates more exogenous DHS into sphingolipids than the *lcb4/5* double mutant.

We could reconstitute the synthesis of ceramide from exogenous DHS using an *in vitro* system that most likely measures the efficient pathway in intact cells, because it requires a phosphorylation/dephosphorylation cycle (Fig. 2). We also demonstrated that Lcb4p and Lcb3p, but not Lcb5p and Ysr3p, mediate this cycle. Surprisingly, the function of Lcb4p could not be bypassed by adding the product of its action, DHS-1P, as a substrate for ceramide synthesis (Fig. 3). We interpret these data as evidence that DHS-1P cannot be incorporated into ceramide if it is formed in the cytosol. Consistent with this idea,

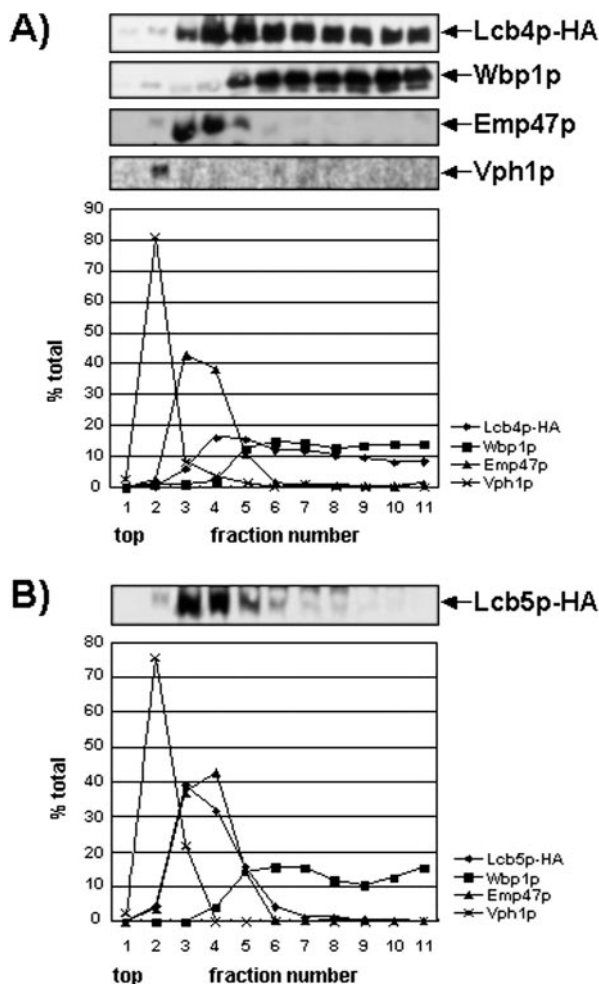


FIG. 7. Lcb4p-HA co-fractionates with both Wbp1p and Emp47p, whereas Lcb5p-HA co-localizes only with Emp47p. *A*, Lcb4p-HA-expressing *lcb5* mutant (RH4950) cells were lysed, and the cleared lysate was loaded onto a 22% to 60% (w/v) sucrose density gradient as described under "Experimental Procedures." The gradient was centrifuged at  $200,000 \times g$  for 2 h and 20 min, and 1-ml fractions were collected from the top. The membrane pellets of fractions were analyzed for the distribution of the indicated proteins by SDS-PAGE, followed by Western blotting. The amount of each protein was quantified by scanning films with a computing densitometer, and the results were expressed as a percentage of total amount. *B*, experiments described in *A* were done with Lcb5p-HA-expressing *lcb4* mutant (RH5257) cells.

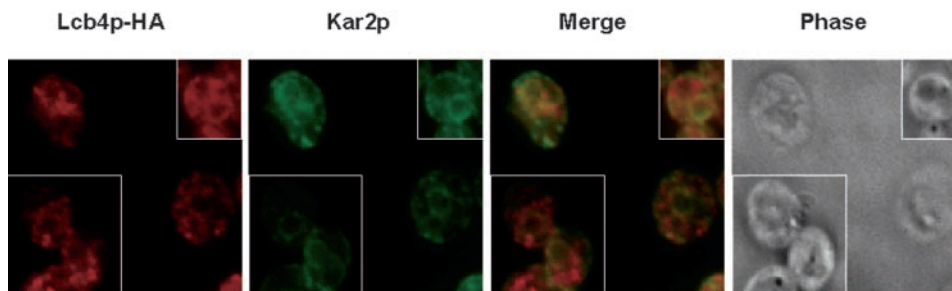


FIG. 8. Lcb4p-HA partially co-localizes with Kar2p. Immunofluorescence analysis was performed as described under "Experimental Procedures." Mutant *lcb5* cells (RH4950) expressing Lcb4p-HA were fixed, converted to spheroplasts, and permeabilized. The cells were then double-stained with anti-HA and anti-Kar2p antibodies. Merged and phase images are also shown.



ceramide synthesis could occur from exogenous DHS when *lcb4/5* mutant cytosol was used in the incubations, but not when *lcb4* and *lcb4/5* mutant membranes were used, even if both LCB kinases were provided by wild-type cytosol extracts (Fig. 2). Further support for a membrane-associated kinase activity and function came from data showing that Lcb4p is the major kinase required for C26-CoA-dependent DH-Cer synthase activity (Fig. 5), as determined by a different *in vitro* system that does not require cytosol (36). This is consistent with fractionation and immunofluorescence data, which indicate that Lcb4p is tightly associated with membranes (Fig. 6) and is partially localized to the ER (Figs. 7 and 8) where Lcb3p dephosphorylates DHS-1P (18). However, the nature of Lcb4p membrane association remains unknown (13).

From the above results we conclude that the LCB kinase Lcb4p must act at the membrane for an effective incorporation of DHS into ceramide. Since the kinase mutant membranes defective for ceramide synthesis had LCBP phosphatase activity to dephosphorylate DHS-1P (Fig. 4), we postulate that a concerted action of Lcb4p and Lcb3p is required for exogenous DHS to enter into the ceramide synthase reaction. The mechanism of this coupling remains unknown for the moment. Several possibilities exist. First, it may be that Lcb4p and Lcb3p form a protein complex. The increased efficiency of the reaction due to the physical association could be necessary to couple the reactions to ceramide synthesis. By using a two-hybrid system, we detected an interaction of Lcb4p with the region containing amino acids 332–384 of Lcb3p (data not shown), which has been predicted to be cytosolically disposed.<sup>2</sup> However, we have not been able to detect a physical association between Lcb4p and full-length Lcb3p by immunoprecipitation experiments. Although this discrepancy remains unknown, it may be explained by the possibility that the Lcb4p-Lcb3p interaction is physiologically weak, transient, and/or indirect. Recently, proteins that interact with SPHK1, a mammalian homologue of Lcb4p, have been identified, and they seem to function as SPHK1 scaffolding or anchoring proteins, which affect the distribution between cytosolic and membrane fractions (41, 42). Second, it could be that Lcb3p is localized throughout the ER, but that it can only couple to ceramide synthase in specialized regions. The discrete staining pattern of Lcb4p-HA in the ER observed by immunofluorescence microscopy (Fig. 8), suggests that Lcb4p could be localized in specialized subdomains of the ER. Association of Lcb4p with these regions could be sufficient to couple the dephosphorylation of DHS-1P to ceramide synthesis. In this case, a direct physical association between Lcb4p and Lcb3p would not be necessary. A precedent for segregation of lipid synthesis reactions in the ER has been found for phospholipid biosynthesis (43). Glycosylphosphatidylinositol biosynthetic reactions are also localized to a subcompartment of the ER, which appears to be similar to the one previously identified for phospholipid biosynthesis as a region of ER associated with mitochondria (44). Moreover, recent evidence indicates that Tsc13p, a protein required for fatty acid elongation, is localized to a specialized domain within the ER membrane at the nuclear-vacuolar interface (45). A third, remote possibility, is that, because the active site of Lcb3p appears to be located in the lumen of the ER,<sup>2</sup> Lcb4p might function as a transporter or regulator to deliver LCBP to the luminal side of ER. Alternatively, Lcb4p might participate directly in regulating ceramide synthase.

Lcb4p represents about 97% of total cellular LCB kinase activity, and the other 3% is due to Lcb5p (13). Because the two cytosolic kinases have similar substrate specificities (13), they

might be expected to have redundant functions. This has been observed for the cell cycle arrest response to heat stress (19). However, our data demonstrate a specific role of Lcb4p in ceramide synthesis from exogenous LCB. Moreover, the deletion of *LCB4* but not the *LCB5* gene prevents growth inhibition and cell death when both Dpl1 lyase and Lcb3 phosphatase pathways are eliminated (20, 21), suggesting a specific role of Lcb4p. We cannot rule out, however, that the apparent specificity is due to Lcb4 kinase being the major activity in the cell. In contrast, Lcb5p but not Lcb4p appears to play a role in heat-stress resistance during induced thermotolerance (22). Thus, it seems that the two LCB kinases have different functions. Interestingly, Lcb4p and Lcb5p show not only different natures of membrane association (Fig. 6) but also different subcellular localizations (Fig. 7). Their different functions could be related to their different cellular locations.

Finally, our studies provide evidence that membrane-associated but not cytosolic Lcb4p is necessary for ceramide synthesis. To our knowledge, this is the first clear case for physiologically distinct roles of membrane-associated and cytosolic kinases. One can speculate that the cytosolic fraction of the LCB kinases form LCBP as a second messenger in response to various stimuli, including heat stress, whereas membrane-associated kinases have distinct roles that might be related to their specific cellular localizations. This can explain why two different kinases have overlapping function, if LCBP, which is generated by either of two cytosolic kinases, can gain access to the target molecule. Consistent with the idea, cytosolic mammalian sphingosine kinases are greatly stimulated by various stimuli containing acidic phospholipids and are inhibited by kinase inhibitors such as *N,N*-dimethyl-sphingosine, but membrane-associated kinases are less sensitive (12, 46), although this may be due to differences in isoforms. Such specialized functions between membrane-associated and cytosolic kinases and/or between the different kinases may exist to properly organize the multiple cellular responses to external and intrinsic signals that lead to changes in signaling lipid metabolites. An additional function might be to funnel excess LCB out of signaling pathways into ceramide synthesis via the ER membrane or into phospholipid biosynthesis via Dpl1p lyase and to spatially separate the production of metabolic intermediates destined for ceramide biosynthesis from the intermediates produced upon stimuli to act as second messengers.

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