Accumulation of Phosphorylated Sphingoid Long Chain Bases Results in Cell Growth Inhibition in *Saccharomyces cerevisiae*

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ABSTRACT

Sphingolipid metabolites in mammals can function as signaling molecules with cell-specific functions. In *Saccharomyces cerevisiae*, phosphorylated long chain bases, such as dihydrosphingosine 1-phosphate and phytosphingosine 1-phosphate, have also been implicated in stress responses. To further explore the biological roles of these molecules, we created disruption mutants for *LCB4*, *LCB5*, *DPL1*, *YSR2*, *YSR3*, and *SUR2*. *LCB4* and *LCB5* encode kinases that phosphorylate long chain bases. *DPL1* and *YSR2*/*YSR3* are involved in degradation of the phosphorylated long chain bases. *SUR2* catalyzes conversion of dihydrosphingosine to phytosphingosine. We adapted an HPLC method to measure intracellular concentrations of the phosphorylated long chain bases. Double mutants of *dpl1* and *ysr2* were inviable, whereas *dpl1 ysr2 lcb4* triple mutants were viable. Further, growth inhibition associated with accumulated phosphorylated long chain bases was observed in the triple mutant *dpl1 ysr2 lcb4* overexpressing *LCB4* or *LCB5*. These results indicate that phosphorylated long chain bases can inhibit cell growth. Mutants defective in both *YSR2* and *SUR2*, which accumulated dihydrosphingosine 1-phosphate only, grew poorly. The phenotypes of the *ysr2 sur2* mutants were suppressed by overexpression of *DPL1*. Our results clearly show that elevated levels of phosphorylated long chain bases have an antiproliferative effect in yeast.

SPHINGOLIPIDS are found in abundance in the membranes of all eukaryotic cells and in some bacteria. Whereas previously they were considered to serve a primarily structural role in membranes, recent studies have indicated that sphingolipids and their metabolic products are highly bioactive compounds that are involved in signal transduction (for a review see Riboni *et al.* 1997).

In mammalian cells, sphingosine 1-phosphate (S1-P) and its metabolic derivative ceramide have been implicated as signaling molecules involved in regulation of cell proliferation, intracellular calcium mobilization, motility, and tumor cell invasiveness (Zhang *et al.* 1991; Sadahira *et al.* 1992; Olivera and Spiegel 1993; Gomez-Munoz *et al.* 1995; Cuvillier *et al.* 1996, 1998; Kupperman *et al.* 2000). These molecules appear to function antagonistically, especially regarding regulation of cell proliferation. For example, reports indicate that ceramide is a mediator of apoptosis and cell cycle arrest (Hannun 1996; Perry and Hannun 1998), while S1-P is implicated in inducing/promoting cell proliferation and inhibition of ceramide-mediated apoptosis (Cuvillier *et al.* 1996, 1998). S1-P is capable of inhibiting activation of caspases induced by Fas or ceramide (Cuvillier *et al.* 1998). Ceramide and S1-P conduct their functions both as intracellular second messengers and as activators of extracellular receptors (van Koppen *et al.* 1996; Lee *et al.* 1998; Zondag *et al.* 1998; Van Brocklyn *et al.* 1999).

Genes encoding the enzymes involved in the synthesis and catabolism of phosphorylated long chain (sphingoid) bases (LCBPs) have been identified in *Saccharomyces cerevisiae* (Figure 1). Therefore, this organism provides an ideal system in which to analyze the function of these molecules. Formation of LCBPs, dihydrosphingosine 1-phosphate (DHS1-P) and phytosphingosine 1-phosphate (PS1-P), is catalyzed by long chain base (LCB) kinases encoded by *LCB4* and its homologue *LCB5* (Nagiec *et al.* 1998). Once formed, two routes of LCBP catabolism exist in yeast. LCBPs can be dephosphorylated back to LCBs by the LCBP phosphatases Ysr2p and Ysr3p (Mao *et al.* 1997; Qie *et al.* 1997; Mandala *et al.* 1998). Alternatively, LCBPs can be cleaved internally at the $C_{2\text{-}3}$ bond by the LCBP lyase Dpl1p, to yield long chain aldehyde and ethanolamine phosphate (Saba *et al.* 1997). Sur2p is required for hydroxylation of dihydrosphingosine at C_4 to yield phytosphingosine (Haak *et al.* 1997).

The biological functions of LCBPs have been examined in the budding yeast *S. cerevisiae*, where studies suggest that they may play a role in cell growth and in the response to heat shock (Gottlieb *et al.* 1999; Skrzypek *et al.* 1999). An increase in the amount of both DHS1-P and PS1-P was observed following heat treatment (Skrzypek *et al.* 1999). In addition, strains lacking *YSR2* or *DPL1* have been shown to display elevated LCBP levels and higher survival after heat shock

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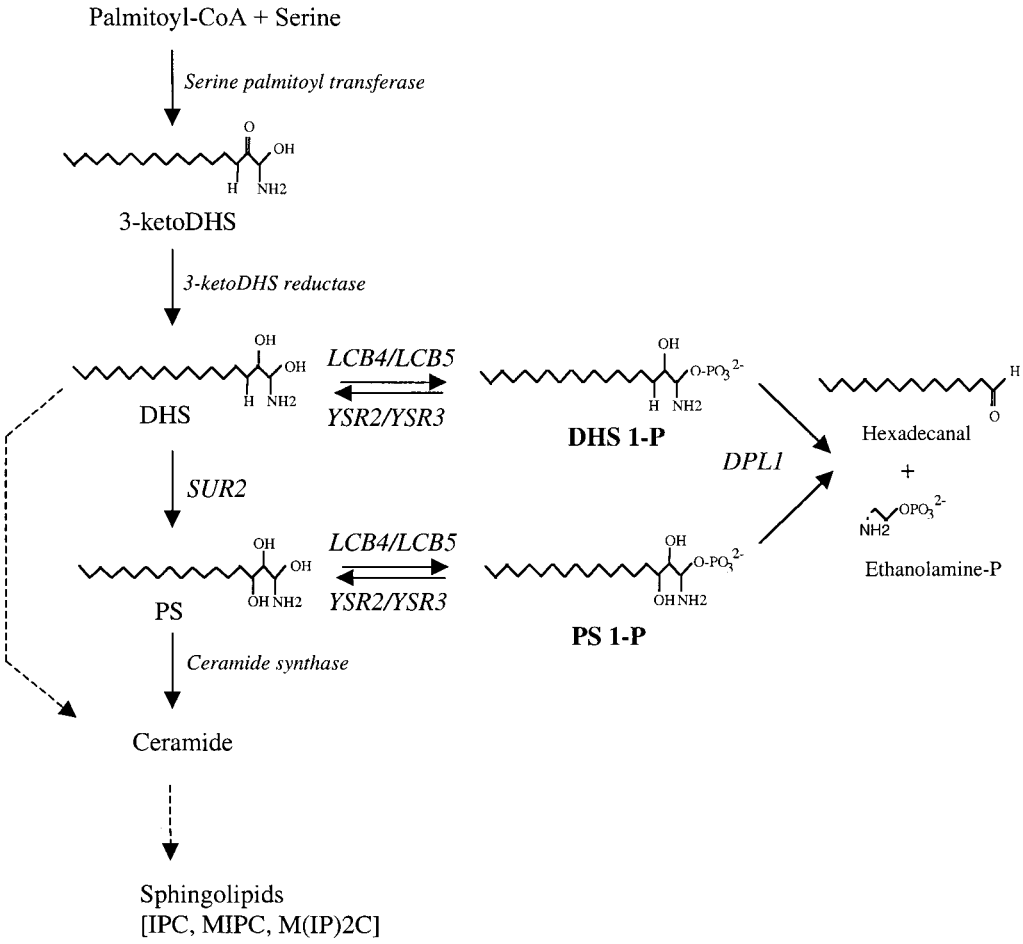


FIGURE 1.—Sphingolipid metabolic pathway proposed in *S. cerevisiae*. *DPL1*, also known as *BST1*, encodes an LCBP lyase (SABA *et al.* 1997). *YSR2*, also independently identified as *LCB3* and *LBP1*, encodes an LCBP phosphatase (MAO *et al.* 1997; QIE *et al.* 1997; MANDALA *et al.* 1998). *YSR3/LBP2/YKR053C* is 53% identical at the amino acid level to *YSR2/LCB3/LBP1*. *LCB4* encodes the major LCB kinase and is 53% identical to *LCB5* (NAGIEC *et al.* 1998). *SUR2*, also independently identified as *SYR2*, encodes LCB hydroxylase (DESFARGES *et al.* 1993; CLIFTEN *et al.* 1996; HAAK *et al.* 1997). Three different but related sphingolipids are found in yeast: inositol-phosphoryl-ceramide (IPC), mannose-inositol-P-ceramide (MIPC), and mannose-(inositol-P)$_2$-ceramide [M(IP)$_2$C].

(SABA *et al.* 1997; MANDALA *et al.* 1998; SKRZYPEK *et al.* 1999). The observed phenotype could be due to LCBP-specific induction of *TPS2* transcription, resulting in the accumulation of the heat-protective storage carbohydrate trehalose (SKRZYPEK *et al.* 1999). Despite data implying a role for LCBPs in some cellular processes induced by endogenous or exogenous stimuli, only limited studies investigating a direct role for LCBPs as second messengers in signal transduction, cell growth, and death in *S. cerevisiae* have been performed to date.

In this article, we describe genetic and HPLC-based biochemical analysis of various mutants defective in the sphingolipid biosynthetic pathway. Through this analysis, we have sought to gain insight into the function of the various enzymes involved in LCBP metabolism in yeast, the effect of intracellular LCBP accumulation, and the roles of LCBPs in yeast biology.

MATERIALS AND METHODS

Media and methods: Yeast media and genetic methods were standard (SHERMAN *et al.* 1986). Yeast genomic DNA was prepared using glass beads and phenol (AUSUBEL *et al.* 1993). To select yeast transformants containing the *Escherichia coli kan*^{r} gene in the *KanMX* module, cells were incubated at least 3 hr in liquid YPD medium (2% yeast extract, 1% peptone, 2% glucose) and then plated on YPD medium containing 200 μg/ml of the drug geneticin (G418; WACH *et al.* 1994). YPG and YPGal media contain 3% glycerol and 2% galactose, respectively, instead of glucose. SC-URA or -LEU medium is synthetic complete medium lacking uracil or leucine, respectively.

Yeast transformations were performed by the LiOAc procedure (ITO *et al.* 1983). The one-step gene disruption or deletion procedures were employed to mutate genes of interest with PCR-amplified disruption constructs (BAUDIN *et al.* 1993; WACH *et al.* 1994; LORENZ *et al.* 1995). The PCR conditions, using r*Tth* DNA polymerase, XL (PE Applied Biosystems, Foster City, CA), were: 1 cycle of 5 min at 94°, 10 cycles of 1 min at 94°, 1 min at 55°, 2 min at 72°, 20 cycles of 1 min at 94°, 1 min at 65°, 2 min at 72° followed by 1 cycle of 10 min at 72°. To amplify disruption constructs containing the *KanMX* module, plasmid pFA6-KanMX2 was used as a template (WACH *et al.* 1994). The primer pairs, which were used to amplify the *KanMX* module flanked by short regions of homology to the target genes, are listed in Table 1. The amplified disruption constructs were designed to replace most of the open reading frames.

Yeast strains: The yeast strains used in this study are listed in Table 2. All are isogenic derivatives of JK9-3d (*leu2-3,112 ura3-52 rme1 trp1 his4 HML***a**) (HEITMAN *et al.* 1991). Most of the strains are meiotic segregants of diploid JK9-3d made singly or multiply heterozygous for disruption constructs *dpl1*Δ*::KanMX*, *ysr2*Δ*::KanMX*, *ysr3*Δ*::KanMX*, *lcb4*Δ*::KanMX*, *lcb5*Δ*::KanMX*, and *sur2*Δ*::KanMX*. To construct diploids from haploids containing *KanMX* only, cells of the opposite mating

TABLE 1

Heterologous primers used to PCR amplify DNA fragments

Construct	Primer sequence
*dpl1*Δ*::KanMX*	Forward: 5′-GGCTAGCTTCTGTAAAGGGATTTTTCCATCTAATACAATGA::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-TACTTGGTGGCGGTATCCTCTCCTGGACCCAACTTGTA::GCATAGGCCACTAGTGGATATG-3′
*ysr2*Δ*::KanMX*	Forward: 5′-GGTAGATGGACTGAATACCTCGAACATTAGGAAAAG::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-CCTTTTGTTTTATGCTATATTTAAGAGGGAAAATAG::GCATAGGCCACTAGTGGATATG-3′
*ysr3*Δ*::KanMX*	Forward: 5′-GACCATTATTCAGACGGTTACTGAATTGGGTGTTACC::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-CTACCTTAAGTTTGTCCAAGTGAAAAAAACTGGGC::GCATAGGCCACTAGTGGATATG-3′
*lcb4*Δ*::KanMX*	Forward: 5′-CCCATCTGATACTTTCCCTTGTCTAACGTACTG::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-CCATACTCCGATGATAGAAACATTATATCGCCT::GCATAGGCCACTAGTGGATATG-3′
*lcb5*Δ*::KanMX*	Forward: 5′-GGTCTCGCCATTCCAGGAAGAAGCAAATAACGTCAGCGA::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-TCTAGTCCCAGCAGGATTGGTGCCATCCTCGTCAACGAG::GCATAGGCCACTAGTGGATATG-3′
*sur2*Δ*::KanMX*	Forward: 5′-TTCTAGTCCGAAGAGGGTGTATACGAAAAGAAAATATACG::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-TGCCTTTACCCAGCAATTGAACGGGAGGTATGCAAAA::GCATAGGCCACTAGTGGATCTG-3′
pGAL-LCB4-pGAL	Forward: 5′-GGTTCCAAGCTTAGC::ATGGTGGTGCAGAAAAAACTTAG-3′
	Reverse: 5′-GGTTCCCTCGAGACA::CTACATGGATTCAAACTCTGT-3′
*Sal*I*-LCB4-Nhe*I	Forward: 5′-GCGGTCGAC::GCAGCGAAAAGTACGCGAAGA-3′
	Reverse: 5′-CGCGCTAGC::CTACATGGATTCAAACTCTGTATC-3′
*Sal*I*-LCB5-Nhe*I	Forward: 5′-GCGGTCGAC::AGTAAGCCAAAAGAGATGAC-3′
	Reverse: 5′-CGCGCTAGC::AGCTCACATAGAATCGAAATCTGTGTC-3′
*Sal*I*-YSR2-Bgl*II	Forward: 5′-GCGGTCGAC::ACAAGCCATTTGTATAG-3′
	Reverse: 5′-CGCAGATCT::GCTATATTTAAGAGGGA-3′
*dpl1*Δ*::URA3*	Forward: 5′-ATGAATTTCCCACAGTTGCCATCCAATGGGATACC::ATTTTTTTTTTATTGTTTTTTTTGATTTCGG-3′
	Reverse: 5′-CTACTTGGTGGCGGTATCCTCTCCTGGACCCCAACTTGATA::GCTTTTTCTTTCCAATTTTTTTTTTTTTCGT-3′
*leu2*Δ*::KanMX*	Forward: 5′-CAAGGATATACCATTCTAATGTCTGCCCCTAAGAAGATCGT::CAGCTGAAGCTTCGTACGC-3′
	Reverse: 5′-AGCAAGGATTTTCTTAACTTCTTCGGCGACAGCATCCA::GCATAGGCCACTAGTGGATATG-3′
*KanMX*Δ*::URA3*	Forward: 5′-GCTAGGATACAGTTCTCACATCACATCCGAACATAAACA::GATTCCGGTTTCTTTGAAAT-3′
	Reverse: 5′-GTCAGTACTGATTAGAAAAACTCATCGAGCATCAAATGA::GGGTAATAACTGATATAATT-3′

Sequences of different origins are separated by double colon (::) marks.

types were mixed on YPD and allowed to mate overnight at 30°. The mixed cells were streaked on YPD and, following 3-day incubation at 30°, a mating type assay was performed on individual colonies to select **a**/α diploids. Haploid strains containing multiple disruptions, each marked with the *KanMX* module, were derived from G418-resistant spores from intact tetrads showing 2:2 segregation for G418 resistance ($G418^r$) and sensitivity ($G418^s$). In tetrad analysis involving two unlinked loci, the average frequency of double mutant spores would be 25%, and tetrads containing two double mutant spores would be encountered, on average, once in every 6 tetrads analyzed. In case of three unlinked loci, such tetrads would occur once in 36 tetrads. In the analysis of a total of 200 tetrads involving *dpl1* and *ysr2*, 187 tetrads showed the following segregation patterns: 29 tetrads contained only two viable spores that were $G418^s$, 32 tetrads contained all four viable $G418^r$ spores, and 126 tetrads contained three viable spores, of which two were $G418^r$ and one $G418^s$ (refer to Figure 3). The proportion of dead spores is $(29 \times 2 + 32 \times 0 + 126 \times 1)/187 \times 4 = 0.25$, and the segregation patterns fit the hypothesis that *dpl1 ysr2* double mutants are inviable ($\chi^2 = 0.1892$, $P \gg 0.05$). All the loci listed above are physically unlinked except *dpl1* (*YDR294C*) and *sur2* (*YDR297W*). The distance between the open reading frames of *DPL1* and *SUR2* is ~4.3 kb. The *dpl1 sur2* double mutants were obtained by transforming a *dpl1*Δ*::URA3* strain with a PCR-amplified

TABLE 2

Yeast strains

Name	Relevant genotype
	Wild-type controls
JSK406	**a** *leu2Δ::KanMX*
JSK437	α *leu2Δ::KanMX*
	Mutants lacking LCBPs
JSK68	**a** *lcb4Δ::KanMX*
JSK69	α *lcb4Δ::KanMX*
JSK382	**a** *lcb5Δ::KanMX*
JSK435	α *lcb5Δ::KanMX*
JSK431	α *lcb4Δ::KanMX lcb5Δ::KanMX*
JSK432	**a** *lcb4Δ::KanMX lcb5Δ::KanMX*
JSK106	**a** *ysr2Δ::KanMX lcb4Δ::KanMX*
JSK107	α *ysr2Δ::KanMX lcb4Δ::KanMX*
JSK436	**a** *dpl1Δ::KanMX lcb4Δ::KanMX*
JSK442	α *dpl1Δ::KanMX lcb4Δ::KanMX*
JSK390	**a** *ysr2Δ::KanMX dpl1Δ::KanMX lcb4Δ::KanMX*
JSK392	**a** *dpl1Δ::KanMX ysr2Δ::KanMX lcb4Δ::KanMX*
JSK398	JSK392 with [*pGAL*-vector *(LEU2)*]
	Mutants accumulating LCBPs
JSK385	α *dpl1Δ::KanMX*
JSK386	**a** *dpl1Δ::KanMX*
JSK14	**a** *ysr2Δ::KanMX*
JSK38	α *ysr2Δ::KanMX*
JSK358	α *ysr3Δ::KanMX*
JSK359	**a** *ysr3Δ::KanMX*
JSK387	α *ysr2Δ::KanMX ysr3Δ::KanMX*
JSK388	**a** *ysr2Δ::KanMX ysr3Δ::KanMX*
JSK444	**a** [*pGAL-LCB4 (LEU2)*]
JSK428	**a** *dpl1Δ::KanMX* [*pGAL-LCB4 (LEU2)*]
JSK448	**a** *ysr2Δ::KanMX ysr3Δ::KanMX* [*pGAL-LCB4 (LEU2)*]
JSK400	JSK392 with [*pGAL-LCB4 (LEU2)*]
JSK402	JSK392 with [*pGAL-LCB5 (LEU2)*]
	Mutants accumulating DHS1-P only
JSK233	**a** *sur2Δ::KanMX*
JSK234	α *sur2Δ::KanMX*
JSK445	**a** *sur2Δ::KanMX* [*pGAL-LCB4 (LEU2)*]
JSK446	α *sur2Δ::KanMX* [*pGAL-LCB4 (LEU2)*]
JSK375	**a** *ysr2Δ::KanMX sur2Δ::KanMX*
JSK376	α *ysr2Δ::KanMX sur2Δ::KanMX*
JSK459	JSK375 with [*pYES2 (URA3)*]
JSK460	JSK375 with [*pYES-DPL1 (URA3)*]
JSK257	**a** *ysr2Δ::URA3 sur2Δ::KanMX*
JSK258	α *ysr2Δ::URA3 sur2Δ::KanMX*
JSK426	α *dpl1Δ::URA3 sur2Δ::KanMX*
JSK306	**a** *dpl1Δ::URA3 sur2Δ::KanMX*
JSK451	α *dpl1Δ::URA3 sur2Δ::KanMX* [*pGAL*-vector *(LEU2)*]
JSK452	**a** *dpl1Δ::URA3 sur2Δ::KanMX* [*pGAL-YSR2 (LEU2)*]
JSK454	α *dpl1Δ::URA3 sur2Δ::KanMX* [*pGAL-LCB4 (LEU2)*]
JSK455D	JSK306 × JSK451
JSK456D	JSK306 × JSK454
JSK457D	JSK426 × JSK452
JSK458D	JSK452 × JSK454

sur2Δ::KanMX construct or by random spore analysis of a diploid doubly heterozygous for *dpl1Δ::URA3* and *sur2Δ::KanMX*. Strains containing *ysr2Δ::URA3* were created by replacing the *KanMX* marker with *URA3* using the *KanMXΔ::URA3* construct (Table 1). The wild-type controls containing the *KanMX* module at the *leu2* locus were obtained from diploids transformed with the *leu2Δ::KanMX* construct. Correct replacement of target genes with disruption sequences was verified by monitoring segregation patterns of the markers and by PCR using three primers: a pair of primers homologous to the immediate upstream and downstream target sequences and a primer with homology to the internal disruption sequence.

To create strains overexpressing *DPL1*, *LCB4*, *LCB5*, or *YSR2*, overexpression plasmids containing the *GAL1, 10* promoter (*pGAL*) were constructed. DNA sequences corresponding to individual genes, tagged with *Sal*I and *Nhe*I (*Bgl*II for *YSR2*) restriction endonuclease recognition sites, were PCR amplified from total genomic DNA using the heterologous primer pairs (Table 1) and cloned into the same sites downstream of the *pGAL* sequence in a YEp51-based integrating vector (pSK185). Next, the final constructs, linearized at the unique *Bst*EII site present within the *LEU2* marker on the vector, were transformed into appropriate yeast strains, and stable integrants were chosen. pYES2-*DPL1* (*pGAL-DPL1*), which was used to overexpress *DPL1*, has been described previously (Saba *et al.* 1997).

Chemicals and compounds: C_{18}-D-*erythro*-Sphingosine 1-phosphate (S1-P) and C_{18}-D-*erythro*-dihydrosphingosine 1-phosphate (DHS1-P) were obtained from Biomol Research Inc. (Plymouth Meeting, PA). C_{18}-D-*erythro*-Sphingosine (S) and C_{18}-D-*erythro*-dihydrosphingosine (DHS) were from Matreya Inc. (Pleasant Gap, PA). C_{18}-D-*erythro*-Phytosphingosine (PS) and AG4-X4 ion-exchange resin were from Sigma Chemical Co. (St. Louis).

Growth conditions for HPLC analysis of LCBPs: Most strains were grown in YPD. Fresh yeast strains were streaked onto YPD plates and grown for 2–4 days at 30°. Colonies were picked from the plates and inoculated into YPD media. Cultures were grown to saturation overnight, rediluted in YPD media to OD_{600} = 0.1, and grown to OD_{600} = 1.0. For overexpression of *LCB4*, *LCB5*, and *YSR2*, cells pregrown in SC-LEU + glucose to saturation overnight were washed twice in sterile water and once in YP, transferred to YPGal medium at OD_{600} = 0.1, and grown to OD_{600} = 0.5 for lipid extraction. For *DPL1* overexpression, cells pregrown in SC-URA + glucose to saturation overnight were washed twice in sterile water and once in SC-URA, transferred to SC-URA + galactose medium at OD_{600} = 0.1, and grown to OD_{600} = 0.5.

Lipid extraction: For the wild-type controls and LCB kinase mutants that are expected to contain low amounts of (or no) LCBPs, 10^{10} cells were harvested to extract lipids. For other strains, 10^8 to 10^9 cells were harvested. Prior to extraction 100 pmol of S1-P was added as an internal standard to the washed cell pellet. Lipids were extracted by adding 5 ml of ice-cold MeOH followed by vortexing for 1 min and tip sonication for 20 sec. Extracts were centrifuged for 5 min in a tabletop centrifuge and the supernatant was collected. A second extraction was performed on the pellet by adding an additional 5 ml of ice-cold methanol followed by vortexing and sonication. The combined lipid extracts were dried down in a speed vac, resuspended in 200 μl of 0.1 M NH_4OH in MeOH, sonicated for 30 sec in a bath sonicator and incubated for 1 hr at 37° to allow for the hydrolysis of esterified acyl chains. Following hydrolysis, the samples were cooled to room temperature, dried down with a flow of N_2 and resuspended in 1 ml of EtOH:H_2O:diethyl ether:pyridine (15:15:5:1; solvent A).

Ion-exchange chromatography: Yeast LCBPs were isolated from other lipids using AG4-X4 chromatography as previously

described (Skrzypek *et al.* 1999). Batch chromatography was used to allow for more convenient sample handling. A total of 500 μl of a solution containing 30% AG4-X4 in solvent A was added to each lipid extract and samples were incubated for 10 min on a shaker. Unbound lipid was removed and the AG4-X4 resin was washed three times with 1 ml of solvent A. LCBPs were eluted three times by an addition of 1 ml of solvent A acidified with glacial acetic acid (8 μl/ml) each time. Eluates were pooled and dried down in a speed vac.

HPLC analysis: LCBPs were derivatized with *ortho*-phthalaldehyde (OPA) as previously described (Caligan *et al.* 2000). The OPA-derivatized LCBPs were separated on a reverse-phase column (Phenomenex, Luna RP-18 3 μ, 4.6 × 75 mm) with the running buffer MeOH; 10 mm potassium phosphate, pH 7.2; 1 m tetrabutylammonium-dihydrogen phosphate (80:19:1 in H_2O). Flow rate was 1 ml/min. The fluorescent LCBP compounds were detected and quantified using a Spectra-Physics (Mountain View, CA) fluorescence detector (SP 8410). The elution peaks corresponding to yeast PS1-P and DHS1-P were identified in several ways: (1) They were identified by comparison to known standards. The retention time of PS1-P was obtained from a LCB kinase assay with PS as a substrate. (2) They were identified by noticing a change in the area of the elution peak of samples run either with or without added PS1-P and DHS1-P. (3) The elution peaks were confirmed by a pH-induced shift in retention times (Caligan *et al.* 2000). The amounts of LCBPs were estimated in comparison to the amount of S1-P. Results were normalized for 1×10^8 cells.

RESULTS

Construction of sphingolipid mutants: To explore the function and metabolism of LCBPs in *S. cerevisiae*, we constructed a set of yeast mutants harboring (singly or in combination) null alleles of genes involved in LCBP metabolism. Previously, however, it has been shown that exogenous phytosphingosine affects uptake of nutrients, notably tryptophan and leucine, by inhibiting corresponding membrane transporters (Skrzypek *et al.* 1998; Chung and Obeid 2000). Consequently, growth of strains with different prototrophic markers even in the same genetic background may well differ in phytosphingosine-containing media. In fact, we observed marker effects of *TRP1* and *URA3* on growth of certain sphingolipid mutants in our strain background even in the standard media containing yeast extract and peptone. The best example is the viability of the *dpl1 ysr2* double disruption mutants; these mutants marked with such nutrient markers as *TRP1*, *URA3*, or *LEU2* are viable though sick (Skrzypek *et al.* 1999; our unpublished results), while those with the bacterial *kan*^{r} gene are inviable (see below). In constructing disruption mutants for this study, therefore, we used the *KanMX* module conferring G418 resistance (Wach *et al.* 1994). The *KanMX* module is known to have no such marker effects (Chung and Obeid 2000; our unpublished results). When we had to use strains with nutrient markers (see below), we did so in parallel with control strains containing the same markers. Accordingly, all the strains described below, unless noted otherwise, are disrupted with the *KanMX* module.

HPLC analysis: Our aim was to determine biological roles, if any, of endogenous LCBPs found in yeast. Toward this end, LCBPs extracted from all the mutants created for this study were derivatized with OPA, and the fluorescent OPA derivatives were separated and quantified by a modified HPLC method that was adapted for yeast samples (see materials and methods). Representative HPLC runs are shown in Figure 2 for the wild-type control (A), *lcb4 lcb5* (B), *ysr2 ysr3* (C), and *ysr2 sur2* (D) mutants. In most cases, peaks corresponding to endogenous PS1-P and DHS1-P were readily identified, along with exogenous S1-P as a control. In other cases, correct peaks were identified by referring to other standards or by shifting the LCBP peaks by changing the pH of the HPLC solvent, as described previously (Caligan *et al.* 2000).

Mutants lacking LCBPs: Previous studies have shown that Lcb4p is responsible for more than 95% of total cellular LCB kinase activity (Nagiec *et al.* 1998). As shown in Table 3, LCBPs were not detected in all mutants defective in *LCB4*, indicating that Lcb4p is responsible for all measurable LCB kinase activity under normal growth conditions. A slight elevation of LCBP levels in the *lcb5* mutants in comparison to the wild-type controls was observed, which implies that Lcb5p may be repressive of the Lcb4p activity, possibly by competing with Lcb4p for the same substrates. Mutants defective in *LCB4*, *LCB5*, or both, grew normally, suggesting that LCBPs are dispensable under normal growth conditions (data not shown).

Unphosphorylated LCBs have been demonstrated to inhibit yeast growth when provided exogenously (Saba *et al.* 1997; Skrzypek *et al.* 1998; Chung and Obeid 2000). Since the LCB-kinase-defective mutants are likely to accumulate LCBs instead of LCBPs, they provide a way of distinguishing the role of intracellular LCBs from that of LCBPs. The LCB levels measured in the *lcb4 lcb5* double and *dpl1 ysr2 lcb4* triple mutants were 12- to 19-fold higher than the wild-type levels (wild-type, 54.79 pmol/10^8 cells; *lcb4 lcb5*, 1044.02 pmol/10^8 cells; *dpl1 ysr2 lcb4*, 652.00 pmol/10^8 cells); nevertheless, the LCB kinase mutants showed no detectable growth defect as mentioned above. Therefore we exclude any role of intracellular accumulation of LCBs in cell growth.

Mutants accumulating LCBPs: Mutants defective in either or both of the LCBP catabolic pathways were also examined. The *dpl1* mutant showed a 4- to 5-fold increase in the PS1-P level and a slight increase in the DHS1-P level. Those mutants defective in *YSR2* or *YSR3* also showed a 4- to 12-fold increase in PS1-P and DHS1-P concentrations. When *ysr3* was combined with *ysr2*, the resulting increase in the LCBP concentrations appeared to be additive. Growth of the *dpl1* or *ysr2 ysr3* mutants was indistinguishable from that of the wild-type controls or the LCB-kinase-defective mutants (data not shown). However, stationary-phase-cell densities of the lyase (5.0×10^8 cells/ml) and phosphatase mutants (5.2×10^8 cells/ml) were significantly higher than that

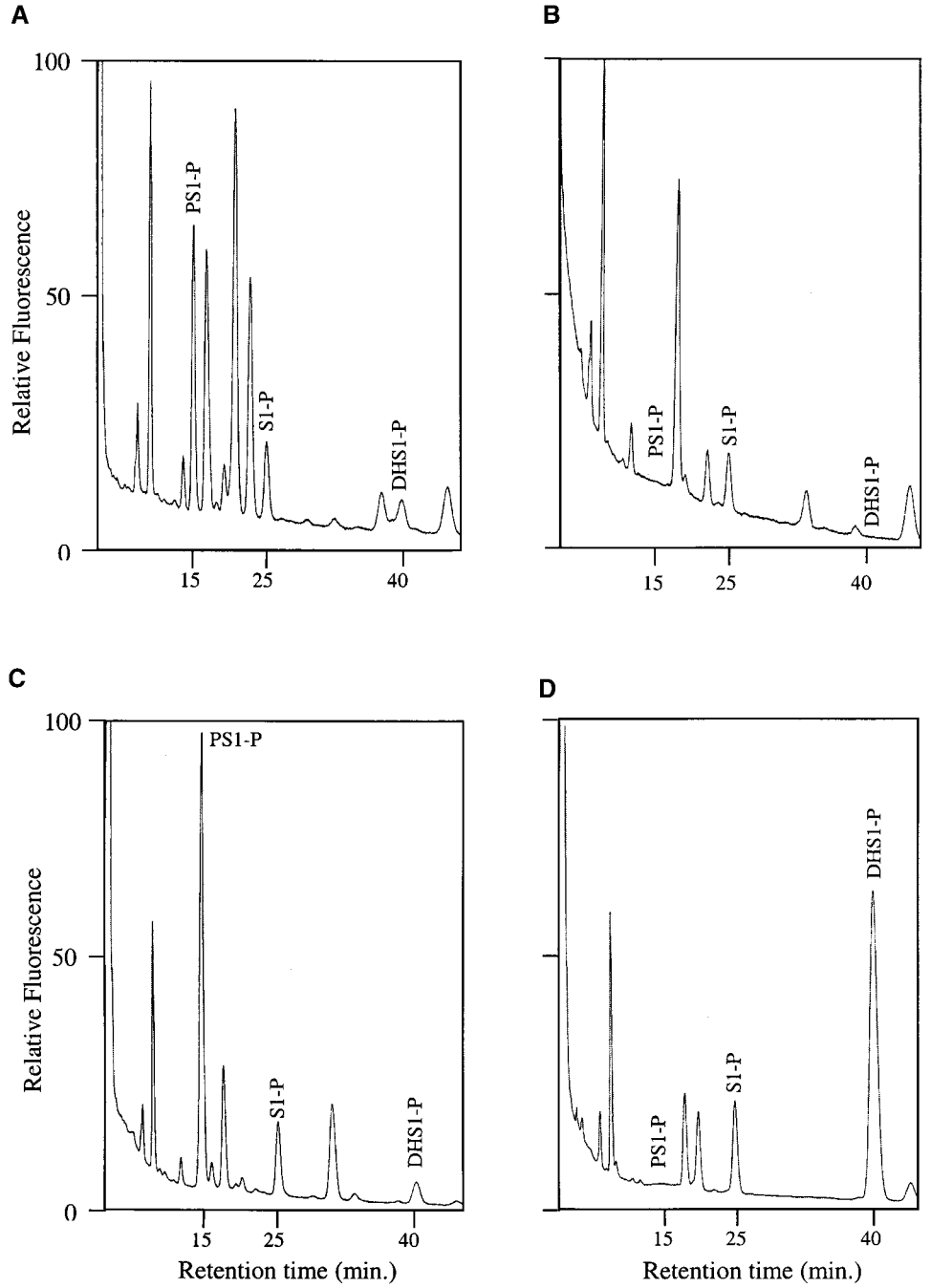


FIGURE 2.—HPLC analysis of LCBPs in the wild type and various mutants. LCBPs were derivatized with *ortho*-phthalaldehyde and resolved on a C_{18} reverse-phase HPLC column, as described in MATERIALS AND METHODS. The fluorescent LCBP compounds were detected with a Spectra-Physics fluorescence detector. Shown are representative elution profiles of (A) 10^{10} cells of wild type (JSK406), (B) 10^{10} cells of *lcb4 lcb5* (JSK432), (C) 10^9 cells of *ysr2 ysr3* (JSK388), and (D) 6×10^8 cells of *ysr2 sur2* (JSK375).

of the *lcb4 lcb5* mutants (4.6×10^8 cells/ml; $P < 0.05$ for both mutants).

To determine the effect of blocking both catabolic pathways, we tried to obtain *dpl1 ysr2* double mutant segregants from double heterozygotes. A total of 200 tetrads were dissected and analyzed, but all the spores that inherited *dpl1* and *ysr2* were found to be inviable (Figure 3; see also MATERIALS AND METHODS). In contrast, triple mutants of *dpl1*, *ysr2*, and *lcb4* were recovered at the expected frequency with no detectable growth defects (Figure 4A). These results indicate that deletion of both *DPL1* and *YSR2* is lethal due to excessive accumulation of excess LCBPs.

Overexpression of *LCB4*/*LCB5* results in growth inhibition in LCBP catabolic mutants: As an approach to understanding how accumulation of LCBPs could affect cell growth and viability, we created derivatives of the *dpl1 ysr2 lcb4* triple mutants in which the major LCB kinase gene *LCB4* or its homologue *LCB5* was expressed under the control of the galactose-inducible *GAL1, 10* promoter. In these strains, cellular levels of LCBPs can be regulated by growing cells in either glucose- or galactose-containing medium. Indeed, when grown in galactose, the triple mutant cells overexpressing *LCB4* or *LCB5* displayed combined LCBP levels more than 500-fold higher than the controls (Table 3). At the same

TABLE 3

HPLC analysis of endogenous C_{18} LCBPs in various strains

Strain	Concentrations in pmol/1 × 10^8 cells[a]	
	DHS1-P	PS1-P
Wild-type control	0.73 ± 0.21 (1.0)	3.18 ± 0.67 (1.0)
Strains lacking LCBPs		
lcb4	0	0
lcb5	1.23 ± 0.61 (1.7)	6.49 ± 1.71 (2.0)
lcb4 lcb5	0	0
lcb4 dpl1	0	0
lcb4 ysr2	0	0
lcb4 dpl1 ysr2	0	0
lcb4 ysr2 sur2	0	0
Strains accumulating LCBPs		
dpl1	1.43 ± 0.73 (2.0)	14.77 ± 2.11 (4.6)
ysr2	7.19 ± 1.27 (9.8)	36.9 ± 3.48 (11.6)
ysr3	3.28 ± 0.47 (4.5)	23.27 ± 2.22 (7.32)
ysr2 ysr3	7.40 ± 3.69 (10.1)	68.16 ± 16.00 (21.4)
dpl1 ysr2 lcb4 [*pGAL-LCB4*]	307.33 ± 211.45 (421.0)	355.92 ± 154.86 (111.9)
dpl1 ysr2 lcb4 [*pGAL-LCB5*]	450.51 ± 275.43 (617.1)	161.67 ± 82.14 (50.8)
Strains accumulating DHS1-P only		
sur2	7.25 ± 0.45 (9.9)	0
ysr2 sur2[b]	72.55 ± 3.21 (99.4)	0
ysr2 sur2 [*pGAL-DPL1*]	48.47 ± 2.70 (66.4)	0
dpl1::URA3 sur2	22.03 ± 2.59 (30.2)	0
dpl1::URA3 sur2 [*pGAL-LCB4*][c]	1057.10 ± 193.69 (1448.1)	0
dpl1::URA3 sur2 [*pGAL-YSR2*][c]	4.41 ± 0.65 (5.7)	0
dpl1::URA3 sur2 [*pGAL-LCB4/pGAL-YSR2*][c]	8.40 ± 0.70 (11.5)	0

[a] LCBP levels were measured by HPLC as described in MATERIALS AND METHODS. Values are shown as mean ± standard deviation for at least three independent measurements. Value 0 means there were no detectable LCBPs at the cell concentrations used. Values in parentheses are ratios of mean values of mutants to those of the wild-type controls.

[b] The measurements for *ysr2*Δ*::KanMX sur2*Δ*::KanMX* are 71.57 and 76.91 (mean = 74.24), and those for *ysr2*Δ*::URA3 sur2*Δ*::KanMX* are 69.23 and 72.49 (mean = 70.86). Since the data are similar, they are pooled for *ysr2 sur2*.

[c] Diploids.

time, growth of these cells was severely retarded in galactose, compared to the control containing the vector only (Figure 4). These data clearly show that intracellular accumulation of LCBPs is growth inhibitory. Overexpression of *LCB4* or *LCB5* in the wild-type controls or in the *dpl1*, *ysr2/3*, *sur2* mutants had no detectable effects on growth with slight increases in the intracellular LCBP levels (data not shown).

DHS1-P alone can inhibit cell growth: To determine whether both DHS1-P and PS1-P are required for the cell growth inhibition, mutants with *sur2* alone or in combination with *ysr2* or *dpl1* were obtained from double heterozygotes. The *SUR2* product catalyzes conversion of DHS to PS (refer to Figure 1). Deletion of *SUR2* resulted in a 10-fold increase in the DHS1-P level, with no detectable change in growth phenotype (data not shown). This indicates either that there exists at least one bypass leading to the synthesis of essential sphingolipids prior to the Sur2p-mediated step or that DHS-based ceramides are sufficient for normal growth. On the other hand, the *ysr2 sur2* segregants, though viable, grew poorly, with about 100-fold increase in the DHS1-P level. The phenotypes of the *ysr2 sur2* mutants were not seen when *LCB4* was additionally disrupted (Figure 5A) or partially rescued by overexpression of *DPL1* (Figure 5B).

In constructing double mutants for the tightly linked *DPL1* and *SUR2*, *URA3* was employed as an additional selection marker. To evaluate the *dpl1 sur2* double mutants containing *URA3*, we first assured ourselves that the poor growth characteristics and high DHS1-P levels of *ysr2*Δ*::URA3 sur2*Δ*::KanMX* and *ysr2*Δ*::KanMX sur2*Δ*::KanMX* were comparable (Figure 6A; Table 3). We then compared the former (JSK306) to the *dpl1*Δ*::URA3 sur2::KanMX* strain. We observed that the *dpl1*Δ*::URA3 sur2* mutants grew much better and contained 3.2-fold less DHS1-P than the *ysr2*Δ*::URA3 sur2* strains. Therefore, we conclude that deletion of *DPL1* and *SUR2* has no measurable effect on cell growth under standard conditions. When *LCB4* was overexpressed in the *dpl1 sur2* cells, growth was inhibited and there was a large increase in the DHS1-P level as expected (Figure 6B; Table 3). These effects of *LCB4* overexpression were

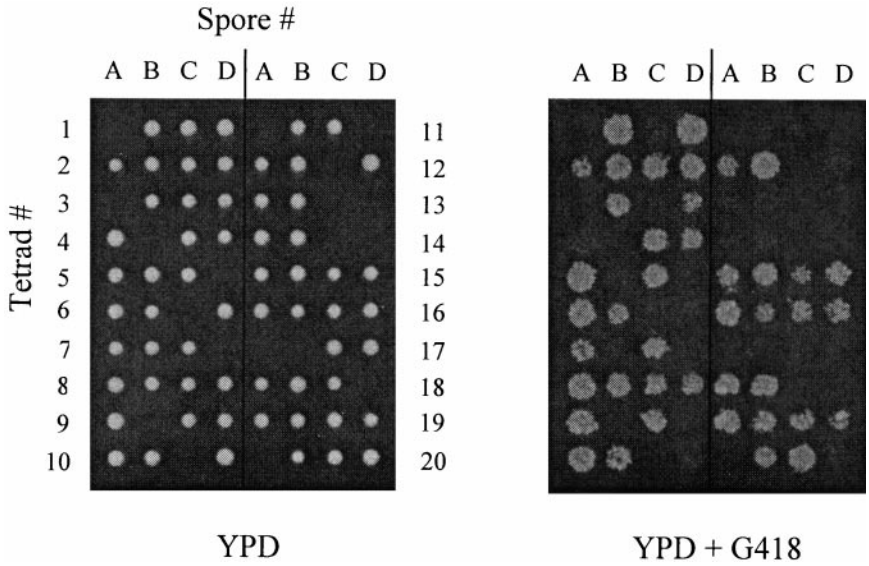


FIGURE 3.—Tetrad analysis of a diploid heterozygous for *dpl1* and *ysr2*. Shown in this figure are 20 tetrads dissected on a YPD plate. Following incubation at 30° for 3 days, the plate was replicated onto YPD medium containing 200 μg/ml G418. Note that there are 4 tetrads with only two viable spores that were $G418^s$, 5 tetrads with all four viable $G418^r$ spores, and 11 tetrads with one $G418^s$ and two $G418^r$ spores. These segregation patterns result from synthetic lethality of the *dpl1Δ::KanMX* and *ysr2Δ::KanMX* mutations. The proportion of dead spores is also 19/80, which is close to 25%.

eliminated when *YSR2* was overexpressed simultaneously (Figure 7).

DISCUSSION

Recently an increasing amount of attention has been directed toward LCBPs for their role as signal molecules in mammals. LCBPs have been reported to be involved in heat-shock responses in *S. cerevisiae* (MANDALA *et al.* 1998; SKRZYPEK *et al.* 1999). Despite the biological significance of these molecules, most of the methods that have been used to analyze intracellular LCBPs rely on the incorporation of a radioactive label and separation of the labeled lipids by thin layer chromatography, which limits accurate resolution and quantification. We have adapted a recently published method based on resolution of fluorescent OPA derivatives of LCBPs by HPLC (CALIGAN *et al.* 2000). Employing this method, we were able to obtain much more reliable and reproducible data regarding the intracellular levels of LCBPs in various yeast strains.

In mammalian cells, DHS1-P is more abundant than PS1-P, both primarily existing as metabolic intermediates in the sphingolipid biosynthetic pathway. However, little is known about the biological roles of DHS1-P and PS1-P. On the other hand, S1-P, which is formed by phosphorylation of sphingosine, has been implicated in a number of important cellular functions including promotion of cell proliferation and inhibition of cell motility, tumor cell invasiveness, ceramide-mediated apoptosis, and developmental processes. Most of these findings were derived from experiments using exogenously added S1-P in medium.

The biological effects of LCBPs in different cell systems can be mediated either (1) through extracellular ligands for specific G protein-coupled cell surface receptors and/or (2) through as yet ill-defined, receptor-independent mechanisms that result from their intracellular accumulation. The former (1) has been shown to be involved in angiogenesis and heart cell migration (LEE *et al.* 1999; KUPPERMAN *et al.* 2000) and probably in some of the effects resulting from exogenous S1-P mentioned above. The latter (2) has been shown responsible for cell proliferation or survival inhibition (OLIVERA *et al.* 1999; SPIEGEL 1999; WANG *et al.* 1999). These findings support the notion that genetic manipulations that result in altered intracellular LCBP levels may have a significant impact on cell growth, death, motility, and migration.

We were interested in obtaining more insight into the biological effects associated with intracellular LCBP accumulation. To do this, we altered the intracellular levels of LCBPs by genetically manipulating the tractable yeast system, and we show in this article that intracellular accumulation of LCBPs has an antiproliferative effect in yeast. This conclusion is drawn from the following observations made by manipulating the LCB kinases and the LCBP catabolic pathways: (1) Double mutants of *dpl1* and *ysr2* were lethal, while *dpl1 ysr2 lcb4* triple mutants were not. (2) Growth of the *dpl1 ysr2 lcb4* triple mutants could be inhibited by *LCB4 or LCB5* overexpression that resulted in increased LCBP levels. Specifically we show that DHS1-P alone is sufficient for the inhibitory effect: (1) *ysr2 sur2* double mutants accumulated DHS1-P and showed poor growth, and these phenotypes could be removed by *LCB4* deletion or by *DPL1* overexpression. (2) Growth inhibition by *LCB4* overexpression in *dpl1 sur2* mutants could be counterbalanced by simultaneous overexpression of *YSR2*. Furthermore, there exists a good correlation between the extent of growth inhibition by LCBPs and their intracellular levels among various mutants. Thus, LCBPs, though inessential, have the potential to affect cell growth under certain physiological conditions leading to their accumulation, whereas

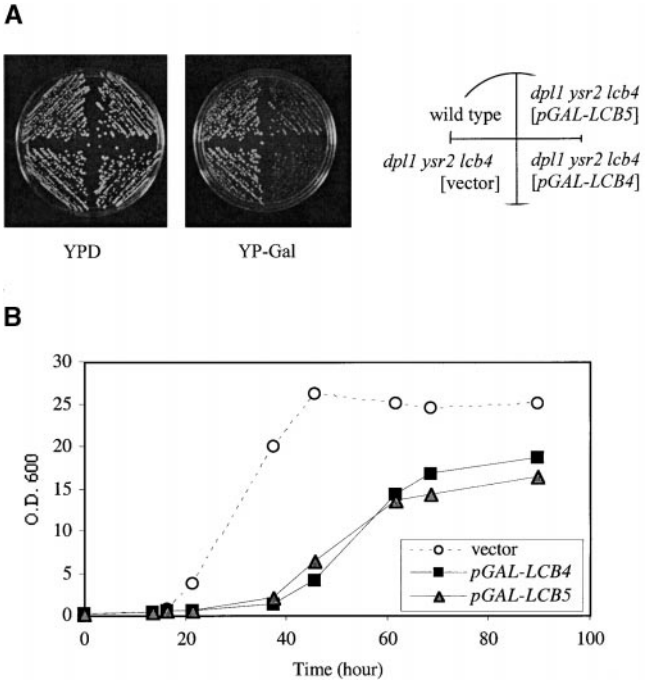


FIGURE 4.—The effect of *LCB4* or *LCB5* overexpression on cell growth. (A) Cells of wild type (JSK406), *dpl1 ysr2 lcb4* [*pGAL*-vector] (JSK398), *dpl1 ysr2 lcb4* [*pGAL-LCB4*] (JSK400), and *dpl1 ysr2 lcb4* [*pGAL-LCB5*] (JSK402) were streaked on a YPD plate. Following 3 days of incubation at 30°, cells were replicated onto a YPGal plate. The YPGal plate was incubated 3 days at 30°. (B) Cells of *dpl1 ysr2 lcb4* [*pGAL*-vector] (JSK398), *dpl1 ysr2 lcb4* [*pGAL-LCB4*] (JSK400), and *dpl1 ysr2 lcb4* [*pGAL-LCB5*] (JSK402) were grown overnight in SC-LEU + glucose. Cells were washed twice in sterile water and once in YP and resuspended in 20 ml of YPGal at a density of $OD_{600} = \sim 0.1$, which corresponds to 2×10^6 cells/ml. Cell densities were monitored by OD_{600} at various timepoints indicated. Shown here is a representative of four independent experiments with similar results.

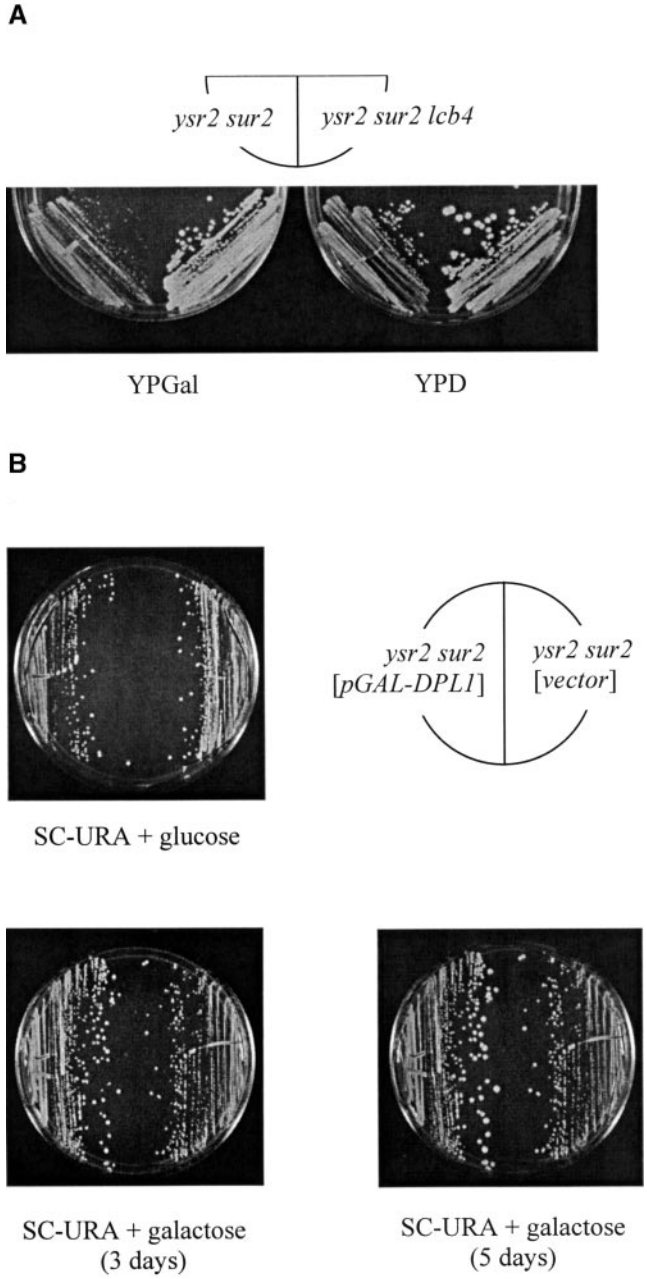
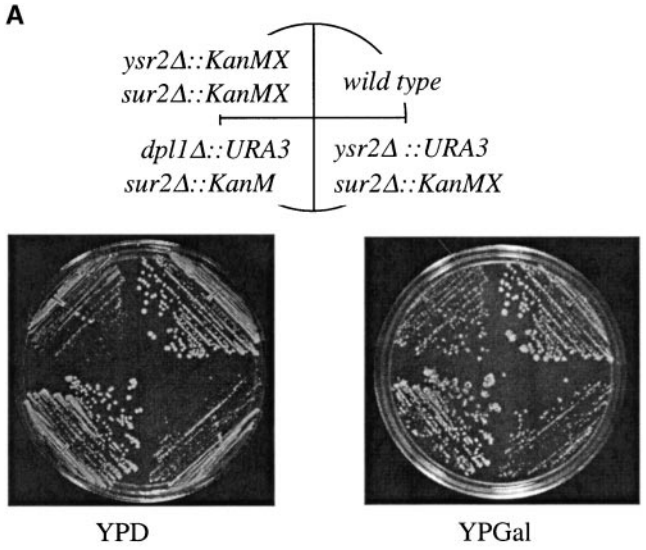


FIGURE 5.—Growth of *ysr2 sur2* strains. (A) Cells of *ysr2 sur2* (JSK375) or *ysr2 sur2 lcb4* (JSK390) were streaked either on a YPD plate or on a YPGal plate and incubated 3 days at 30°. Note that the *ysr2 sur2* cells forming much smaller colonies on the YPGal plate are not petite because petites in the JK9-3d strain background do not grow on galactose-containing medium (our unpublished results). (B) Cells of *ysr2 sur2* with either *pGAL-DPL1* (JSK460) or *pGAL*-vector (pYES2; JSK459) were grown overnight in SC-URA + glucose, washed twice in sterile water, and streaked onto the plates indicated above. The plates were incubated 3 or 5 days at 30°.

accumulation of LCBs had no effect. Of course the actual physiological threshold levels of LCBPs required for the inhibitory effect are difficult to measure on the basis of our available data. Considering the phenotypes of the double mutants *ysr2 sur2* with or without *DPL1* overexpression, however, it can be estimated to be about 70 times the wild-type level in our strain background (Table 2).

A "moderate" increase (*i.e.*, ≥ 70-fold) in LCBP levels was observed in the strains with *ysr2*, *dpl1*, *ysr2 ysr3*, or *dpl1 sur2* mutations. Under normal growth conditions, a moderate increase in the LCBP level appears to have little effect on cell growth, as shown by the growth phenotype of these mutants. A number of studies, however, suggest that the moderate LCBP increase may play a role in mediating protective stress responses. For example, a good correlation exists between moderately elevated LCBP concentrations and enhanced cell survival following heat shock. A 5- to 8-fold surge in the LCBP level was observed when cells were exposed to a higher temperature (SKRZYPEK *et al.* 1999). Deletion of *YSR2* resulted in a moderate increase in LCBP levels and the *ysr2* cells survived better upon heat shock, while *YSR2* overexpression resulted in a lower DHS1-P level and a higher heat-shock sensitivity (MANDALA *et al.* 1998; MAO



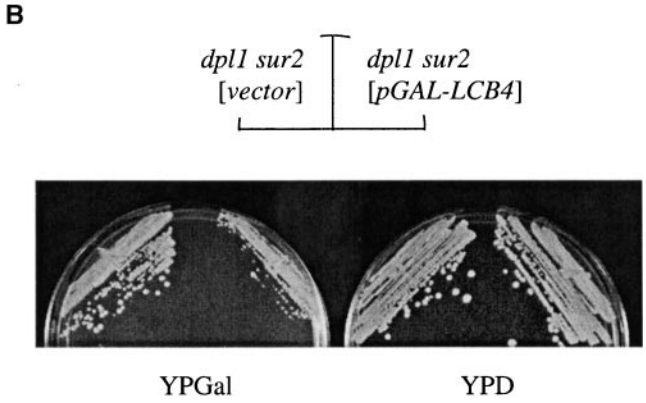


FIGURE 6.—Growth of *dpl1Δ::URA3 sur2* strains. (A) Cells of wild type (JSK406), *ysr2Δ::KanMX sur2Δ::KanMX* (JSK375), *ysr2Δ::URA3 sur2Δ::KanMX* (JSK257), and *dpl1Δ::URA3 sur2Δ::KanMX* (JSK306) were streaked on a YPD plate. Following incubation for 3 days at 30°, cells were replicated onto a YPGal plate. The YPGal plate was also incubated for 3 days at 30°. (B) Cells of *dpl1Δ::URA3 sur2Δ::KanMX* with *pGAL*-vector (JSK451) or with *pGAL-LCB4* (JSK454) were grown overnight in SC-LEU + glucose, washed twice in sterile water, and streaked onto the plates indicated above. The plates were incubated 3 or 5 days at 30°.

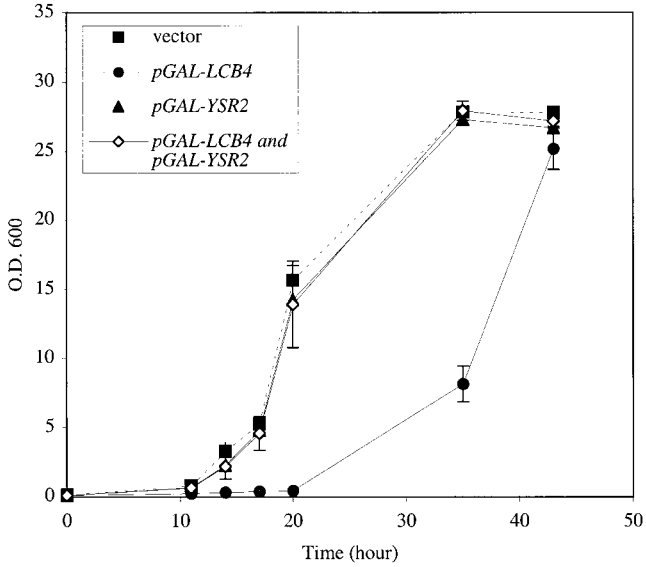


FIGURE 7.—The effect of *LCB4* or *YSR2* overexpression on growth of *dpl1 sur2* double mutants. Diploid cells containing *pGAL*-vector (JSK455D), *pGAL-LCB4* (JSK456D), *pGAL-YSR2* (JSK457D), or both *pGAL-LCB4* and *pGAL-YSR2* (JSK458D) at the *leu2* locus were grown overnight in SC-LEU + glucose. Cells were washed twice in sterile water and once in YP and resuspended in 20 ml of YPGal at a density of OD_{600} = ~0.1. Cell densities were monitored as described in Figure 4B.

and OBEID 2000). These data implicate the moderate increase in the LCBP level as a mediator of the stress response. One of the possible thermoprotective mechanisms elicited by LCBPs is induction of *TPS2* encoding a trehalose synthase, which occurred almost at the same time as the increase in the LCBP levels following heat treatment (SKRZYPEK *et al.* 1999). It is also possible that other gene products involved in the heat-shock response, such as heat-shock proteins, may also participate in the LCBP-mediated protective mechanisms. In this regard it is interesting to note the significantly higher cell densities displayed by the *ysr2 ysr3* double and *dpl1* single mutants during the postdiauxic phase. Stationary phase cells are known to be more resistant to various stresses including heat shock (reviewed by WERNER-WASHBURNE *et al.* 1993).

In sum, we suggest that LCBPs in yeast play a dual role depending on their cellular concentrations. At moderate concentrations, LCBPs may act as beneficial signal transducers involved in mediating protective stress responses. At high concentrations, on the other hand, they are antiproliferative. Studies are under way to elucidate the mechanism for the role of LCBPs in signal transduction and the role of LCBPs in growth inhibition in yeast.

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