The *PLB2* Gene of *Saccharomyces cerevisiae* Confers Resistance to Lysophosphatidylcholine and Encodes a Phospholipase B/Lysophospholipase†

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ABSTRACT: The *PLB1* gene of *Saccharomyces cerevisiae* encodes a protein that demonstrates phospholipase B, lysophospholipase, and transacylase activities. Several genes with significant homology to *PLB1* exist in the *S. cerevisiae* genome, raising the possibility that other proteins may contribute to the total phospholipase B/lysophospholipase/transacylase activities of the cell. We report the isolation of a previously uncharacterized gene that is highly homologous to *PLB1* and that, when overexpressed, confers resistance to 1-palmitoyllysophosphatidylcholine. This gene, which is located adjacent to the *PLB1* gene on the left arm of chromosome XIII and which we refer to as *PLB2*, encodes a phospholipase B/lysophospholipase. Unlike *PLB1*, this gene product does not contain significant transacylase activity. The *PLB2* gene product shows lysophospholipase activity toward lysophosphatidylcholine, lysophosphatidylserine, and lysophosphatidylethanolamine. Whereas deletion of either *PLB1* or *PLB2* resulted in the loss of 80% of cellular lysophospholipase activity, a *plb1/plb2* double deletion mutant is completely devoid of lysophospholipase activity toward the preferred substrate lysophosphatidylcholine. Overexpression of *PLB2* was associated with an increase in total cellular phospholipase B/lysophospholipase activity, as well as the appearance of significant lysophospholipase activity in the medium. Moreover, overexpression of *PLB2* was associated with saturation at a higher cell density, and an increase in total cellular phospholipid content, but no change in phospholipid composition or fatty acid incorporation into cellular lipids. Deletion of *PLB2* was not lethal and did not result in alteration of membrane phospholipid composition or content. *PLB2* gene expression was found to be maximal during exponential growth conditions and was decreased in late phase, in a manner similar to other genes involved in phospholipid metabolism.

In the budding yeast *Saccharomyces cerevisiae*, as in higher eukaryotes, phospholipids serve as vital structural components of cellular membranes (*1*−*4*) and also as a reservoir for the generation of second messengers involved in signal transduction pathways (*5*−*10*). Phospholipid composition of yeast membranes is rigidly maintained during exponential growth under favorable conditions (excess fermentable carbon source, nitrogen source, and 30 °C). However, it is changed markedly in response to availability of exogenous soluble phospholipid precursors, nutrient deprivation, ethanol exposure, osmotic stress, oxygen depletion, and high temperature (*11*−*17*). It is, thus, not surprising that specific phospholipases have been implicated in the regulation of numerous cellular processes in yeast, including growth, secretion, meiosis, respiration, the initiation of mitotic cell division, and nutritional and stress responses (*5*, *18*−*23*). Several phospholipase activities and some of the genes encoding them have been identified in yeast, including phospholipases A_1, A_2, B, C, and D. Other genes with significant nucleotide homology to known phospholipases have been identified through the completed *Saccharomyces* genome sequencing project (*23*−*27*). Thus, *S. cerevisiae* serves as a useful model system in which to identify genes involved in phospholipid metabolism.

Glycerophospholipids in yeast can be degraded and remodeled through the actions of phospholipases, lysophospholipases, transacylases, and acyltransferases. Phospholipase B activity, encoded by the *S. cerevisiae PLB1* gene, catalyzes the deacylation of glycerophospholipids at both *sn*-1 and *sn*-2 positions (*24*). In addition, the *PLB1* gene product exhibits lysophospholipase and transacylase activities, the former that catalyzes the deacylation of lysophospholipids and the latter that catalyzes transfer of an acyl chain from one lysophospholipid to another, allowing the resynthesis of glycerophospholipids (Figure 1). Several genes with significant homology to *PLB1* exist in the *S. cerevisiae* genome, raising the possibility that other proteins may contribute to the total phospholipase B/lysophospholipase/transacylase activities of the cell. In this study, we have identified a second phospholipase B/lysophospholipase through a high-copy screen for genes that confer resistance to toxic concentrations of 1-palmitoyllysophosphatidylcholine (lysoPC). In contrast to *PLB1*, with which it shares 64% nucleotide identity, this gene, which we refer to as *PLB2*, encodes a protein that contains no significant transacylase activity. The biochemical characteristics of a *PLB2* overexpression and deletion strain are examined.

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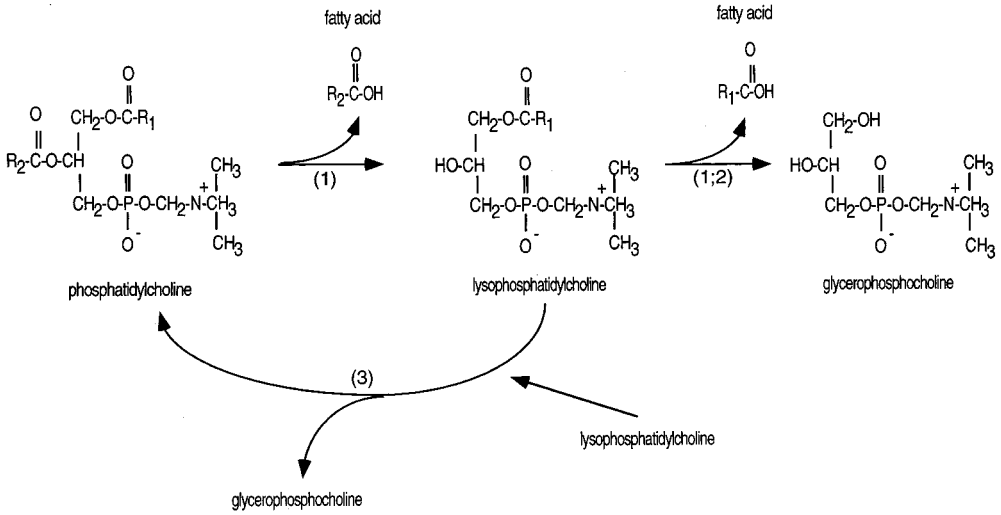

FIGURE 1: Plb1p activity toward phospholipid substrates containing a choline headgroup. Phospholipase B (1), lysophospholipase (2), and transacylase (3) activities are represented.

Table 1: Yeast Strains

designation in this paper	strain	genotype	source
wt	DG338	MATα *his3*Δ200 *ura 3−167 GAL*	ref *28*
wt	DG340	MATa *his3*Δ200 *lys 2 ura 3−52 trp1*	ref *28*
Δ*plb2*	JS100	DG338 *plb2*Δ::NEO	this work
Δ*plb1*	JS101	DG340 *plb1*Δ::HIS3	this work
Δ*plb1*/Δ*plb2*	JS102	*plb1*Δ::*HIS3 plb2*Δ::NEO	this work
PLB1$^+$	JS103	DG338 with pRSP1 (pRS202 containing a 6.8-kb insert containing *PLB1*)	this work
PLB1$^+$/*PLB2*$^+$	JS104	DG338 with pRSP2 (pRS202 containing a 7.3-kb insert containing both *PLB1* and *PLB2*)	this work
PLB2$^+$	JS105	DG338 with pRSP2 with a 1.3-kb *Eco*RI deletion in *PLB1*	this work

EXPERIMENTAL PROCEDURES

Materials. 1-[^{14}C]PalmitoyllysoPC[1] and [^{14}C]oleyl-CoA were purchased from Amersham Corp., Arlington Heights, IL. 1,2-[^{14}C]Dipalmitoyl-PC, [^{14}C]palmitate, and Gene Screen Plus membrane were purchased from DuPont−New England Nuclear Corp., Boston, MA. PC, 1-palmitoyllysoPC (lysoPC), 1-palmitoyllysophosphatidylserine (lysoPS), 1-palmitoyllysophosphatidic acid (lysoPA), 1-palmitoyllysophosphatidylethanolamine (lysoPE), 1-palmitoyllysophosphatidic acid (lysoPA), bovine liver lysophosphatidylinositol (lysoPI) were obtained from Avanti Polar Lipids Inc., Alabaster, AL. Silica plates for thin-layer chromatography were obtained from Analtech Inc., Newark, DE. Palmitic acid, heptadecanoic acid, zymolase, chymostatin, leupeptin, antipain, pepstatin, glucose and galactose were products of Sigma Chemical Co., St. Louis, MO. Yeast extract, peptone, and agar were products of Difco Laboratories, Detroit, MI. pYES2 yeast shuttle vector was obtained from Invitrogen, Inc., Carlsbad, CA. Oligonucleotide primers were synthesized by Operon Technologies, Inc., Alameda, CA. All other compounds used were reagent-grade.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GPI, glycosylphosphatidylinositol; LAT, acyl-CoA lysophosphatidylcholine acyltransferase; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LysoPC, 1-palmitoyllysophosphatidylcholine; LysoPE, 1-palmitoyllysophosphatidylethanolamine; LysoPS, 1-palmitoyllysophosphatidylserine; LysoPA, 1-palmitoyllysophosphatidic acid; LysoPI, bovine liver lysophosphatidylinositol; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; YEPD, yeast extract peptone dextrose; 5′-FOA, 5′-fluoroorotic acid.

Yeast Strains and Growth Conditions. The *S. cerevisae* strain DG338 (Matα *his3*Δ*200 ura3-167 GAL*) and the closely related strain DG340 (Mat**a** *lys2 his3*Δ200 *ura3-52 trp1*) were a kind gift of David Garfinkel (See Table 1) (*28*). All gene deletions were created through homologous recombination with a PCR-generated fragment for targeted replacement of the wild-type allele with a deletion allele containing a selectable marker (*29*). The heterologous primers used to generate such DNA fragments are as follows: *PLB2::NEO* heterologous 5′ primer, 5′-CGCAGATGCAATTACGGAACATATTACAGGCTAGCCAGCTGAAGCTTCGTACGC-3′; *PLB2*::*NEO* heterologous 3′ primer, 5′-GATTGCAGAGGCAATAGCACCGCTTTGATACGTACGCATAGGCCACTAGTGGATCTG-3′; *PLB1::HIS* heterologous 5′ primer, 5′-GTTGCAGAGTTTGTTGGTTTCTGCTGCAGTTTTGACCCACCTAGCGGATGACTCTTTTTTTTTCTTAGCGA-3′; *PLB1*::*HIS* heterologous 3′ primer, 5′-TTAGACCGAAGACGGCACTAATGACACTTAAGACACCTGCTGCAGCTTTAAATAATCGGTGTCACTA-3′. The pRS202 2μ yeast genomic library was used in the screen for high-copy suppressors of lysoPC toxicity (*30*). Strains were grown in yeast extract peptone dextrose (YEPD) medium at 30° C with agitation for the designated time period prior to analysis. Plates containing lysoPC were made by addition of sterile lysoPC to cooled YEPD agar medium. Dilutional assays on lysoPC were performed as previously described (*31*). Selection for plasmid loss was performed by allowing growth on plates containing 5′-fluoroorotic acid (5′-FOA), as previously described (*32*).

Preparation of Cell Extracts. Yeast cells were grown to exponential or late phase and harvested by centrifugation.

Cytosolic extracts were prepared by collecting 2×10^9 cells, washing in 10 mL of H_2O, and resuspending in 1 mL of lysis buffer (50 mM potassium phosphate, pH 7.2, containing 10% glycerol, 2 mM EDTA, 1 mM PMSF, and 10 μg/mL each chymostatin, leupeptin, antipain, and pepstatin). Glass beads (800 μL) were added, and cells were vortex-mixed at 4 °C. Cell extract was recovered with a narrow glass pipet. Remaining glass beads and larger cell structures were removed from recovered cell extract by centrifugation. The supernatant was frozen on dry ice/ethanol and stored at −80 °C until used. Periplasmic extracts were prepared by treating cells with zymolyase as described (*33*). After 40 min of incubation at 30 °C, samples were sedimented at 6000*g* to remove spheroplasts, followed by centrifugation at 50000*g* for 30 min. The resulting clear supernatant was stored at −80 °C until used.

Measurement of Phospholipase Activity. Phospholipase B, lysophospholipase, and transacylase activity associated with the various yeast strains or the conditioned media was assayed following the procedure of Witt et al. (*34*). Radiolabeled substrates used were either 1,2-[^{14}C]dipalmitoyl-PC or 1-[^{14}C]palmitoyllysoPC. Lipids were extracted by adding four volumes of $CHCl_3$/MeOH (2:1). Organic phase was removed and dried under nitrogen. Lipids were redissolved in a small volume of $CHCl_3$/MeOH (2:1) and applied to a silica gel TLC plate. The plates were developed in $CHCl_3$/CH_3OH/CH_3COOH/0.9% NaCl (100:50:16:5). PC, lysoPC, and fatty acid were scraped off and radioactivity was determined in a liquid scintillation counter. In assays with nonradiolabeled lysophospholipid substrates, lysophospholipase activity was determined by measuring the amount of formed free fatty acids by gas chromatography (*35*). Heptadecanoic acid was used as an internal standard.

Measurement of Acyltransferase Activity. Acyl-CoA lysophospholipid acyltransferase (LAT) activity was measured by following the formation of labeled PC from [^{14}C]oleyl-CoA and lysoPC as previously described (*36*). PC was isolated and radioactivity was determined as described above.

Plasmids, Libraries, and Molecular Biology Techniques. DNA sequence analysis was performed by the dideoxynucleotide sequencing method (*37*). Subcloning, restriction enzyme analysis, bacterial transformations, and plasmid DNA isolation from bacterial cells were performed by standard procedures as described (*38*). Yeast cells were transformed with the yeast genomic pRS202 library by the lithium acetate procedure (*39*). Plasmid isolation from yeast was performed as described (*40*).

Analysis of Phospholipid Content. Yeast cells were harvested by centrifugation. Extraction of lipids was performed by the method described by Desfarges et al. (*41*). Lipids were applied to a silica gel TLC plate. Lipids were separated using 2-dimensional TLC as described (*42*). Phospholipid classes were normalized to inorganic phosphate as described by Rouser et al. (*43*).

Lipid Uptake and Metabolism. Strains DG338, DG340, JS100, JS101, JS102, and JS105 were grown to an optical density at 600 nm of 1 in 3 mL cultures of minimal medium (uracil$^-$ medium for JS105, complete medium for all other strains). Either [^{14}C]palmitate or 1-[^{14}C]palmitoyllysoPC (specific activities were 0.3 nCi/nmol) was added to each culture at a final concentration of 50 μM, and cultures were incubated with agitation in glass tubes for 3−4 h. Cells were harvested by centrifugation and washed with a solution of 0.2% fatty acid free bovine serum albumin. Lipids were extracted and separated on two-dimensional TLC by the methods described above. Total radioactivity contained in both cell pellet and medium as well as radioactivity associated with lipid spots from the TLC were determined by liquid scintillation counting.

Lysophosphatidylcholine Sensitivity Assay. Strains DG338, DG340, JS100, JS101, JS102, and JS105 were grown to saturation and diluted to an optical density at 600 nm of 0.2 in minimal medium containing varying concentrations of lysoPC. Cultures were briefly sonicated, and growth was monitored by turbidity measurements at $OD_{600\ nm}$.

Preparation of Total RNA and RNA Blot. Total RNA was prepared by the method described by Ellwood and Craig (*44*). The RNA samples (15 μg/lane) were separated by formaldehyde agarose gel electrophoresis and transferred to a Gene Screen Plus membrane according to the manufacturer's instructions (New England Nuclear). Ethidium bromide staining was used to ensure uniform loading. The blots were probed with a 0.3 kb DNA fragment generated by PCR with the following primer pair: 5′-CTAAGTACCTCAGCTAATC-CTG-3′ and 5′-TTCGCATAGGAAATAGCCGCAGC-3′. This primer pair amplifies a 305 bp DNA fragment at the 3′ end of the *PLB2* open reading frame. This stretch of DNA sequence is unique to *PLB2* and nonhomologous to *PLB1* or *YOL011w*. Probe was radiolabeled with ^{32}P by random primer labeling (Gibco−BRL). Blots were analyzed with a Molecular Dynamics PhosphorImager.

RESULTS

Selection for LysoPC Resistance. We were interested in determining whether yeast genes involved in phospholipid catabolism and remodeling could be isolated by a genetic screen in which the survival of the cell depended on metabolism of excess phospholipid substrate. Toward this end, we investigated the effects of different concentrations of lysoPC on the growth of yeast cells on YEPD agar plates. We found that high concentrations of lysoPC inhibited colony formation. The effects of lysoPC were dependent upon the number of cells plated, consistent with its probable distribution among cell membranes. A 10 mM concentration of lysoPC was sufficient to inhibit all colony formation on plates containing 1.8×10^3 cells.

To isolate genes that suppress the toxicity of lysoPC, we then transformed wild-type yeast with a yeast genomic library in the high-copy vector pRS202. This library allows the overexpression of yeast genes utilizing their native promoters by virtue of their presence on a plasmid containing the yeast plasmid 2μ origin of replication. Transformants were isolated by selection for uracil prototrophy. Transformants were then plated at 10^3 cells/90 mm plate onto YEPD agar plates containing 10 mM lysoPC. Over 20 clones with significant resistance to lysoPC were obtained. Ten plasmids that conferred lysoPC resistance to wild-type cells and that were required for resistance as demonstrated by loss of resistance upon selection for plasmid loss were chosen for further study.

PLB1 and PLB2 Are LysoPC Resistance Genes. The lysoPC-resistant clones comprised two categories, based upon restriction analysis. Partial DNA sequence analysis was performed on representative members of each of the two

groups. The first group contained plasmids carrying a small DNA insert, from the left arm of *Saccharomyces* chromosome XIII, on which only a single complete open reading frame, *PLB1*, was present. Identification of *PLB1* as a lysoPC resistance gene was not unexpected, since overexpression of *PLB1* would be anticipated to increase the rate of deacylation of lysoPC and allow its rapid removal from the medium. Interestingly, the second group contained an insert that, in addition to *PLB1*, carried a second complete open reading frame that was located adjacent to *PLB1* on chromosome XIII and was identified in the *Saccharomyces* genome database as YMR006c (*45*). This open reading frame, which we designate *PLB2*, encodes a predicted protein of 706 amino acids, with a deduced molecular mass of 75.5 kDa and an isoelectric point of 4.35. *PLB2* demonstrated 63.9% identity with *PLB1*, indicating that it was likely to have been created through a gene duplication event. The homology to *PLB1* spanned the length of the *PLB2* gene, with the exception of 305 nucleotides near the 3′ end of the open reading frame. *PLB2* contained a stretch of 16 hydrophobic amino acids and a predicted GPI-anchor cleavage motif at the C-terminus (*46*). In addition, the first 20 amino acids at the N-terminus conform to the properties of a signal peptide, indicating that Plb2p is likely to be a secreted protein (*47*).

To determine whether *PLB2* overexpression was sufficient to confer resistance to lysoPC in the absence of *PLB1*, a 1.3 kb deletion spanning the majority of the *PLB1* coding region was created by *Eco*RI digestion, leaving a plasmid (pRSP2) with only the *PLB2* open reading frame intact. This construct, which we refer to as *PLB2*$^+$, conferred resistance to lysoPC, confirming that *PLB2* overexpression was sufficient to confer resistance to lysoPC and that *PLB2* was likely to encode a phospholipase B/lysophospholipase (Figure 2, top). Interestingly, when colonies of the *PLB2*$^+$ strain were grown on YEPD plates containing lysoPC, a large halo of material was deposited around each *PLB2*$^+$ colony, suggesting lysoPC hydrolysis and fatty acid accumulation as a result of enzyme secretion (Figure 2, bottom).

Enzyme Activities of PLB1 and PLB2 toward PC and LysoPC. To explore the function of *PLB2* and its relationship to *PLB1*, we created single Δ*plb1* and Δ*plb2* deletion strains and also a Δ*plb1*/Δ*plb2* double deletion strain using PCR-generated fragments containing either the kanMX kanamycin-resistance module or a *HIS3* selectable marker. We then evaluated the phospholipase B, lysophospholipase, and transacylase activities of wild-type strain DG338, the Δ*plb1*, Δ*plb2*, and Δ*plb1*/Δ*plb2* deletion strains, and the strains overexpressing *PLB1* and *PLB2* (which we will refer to as *PLB1*$^+$ and *PLB2*$^+$, respectively) toward PC and lysoPC substrates. As can be seen in Table 2, strains overexpressing *PLB1* and *PLB2* had a 10-fold increased phospholipase B/lysophospholipase activities when compared to the control strain, indicating that both genes encode phospholipase B/lysophospholipases. These activities were appreciated in both the cell extracts and within the culture medium, indicating either secretion of enzyme or failure to properly localize all overexpressed protein to the membrane. Deletion of either *PLB1* or *PLB2* led to marked loss of cellular phospholipase B/lysophospholipase activities, and the Δ*plb1*/Δ*plb2* strain contained no appreciable phospholipase B/lysophospholipase activity.

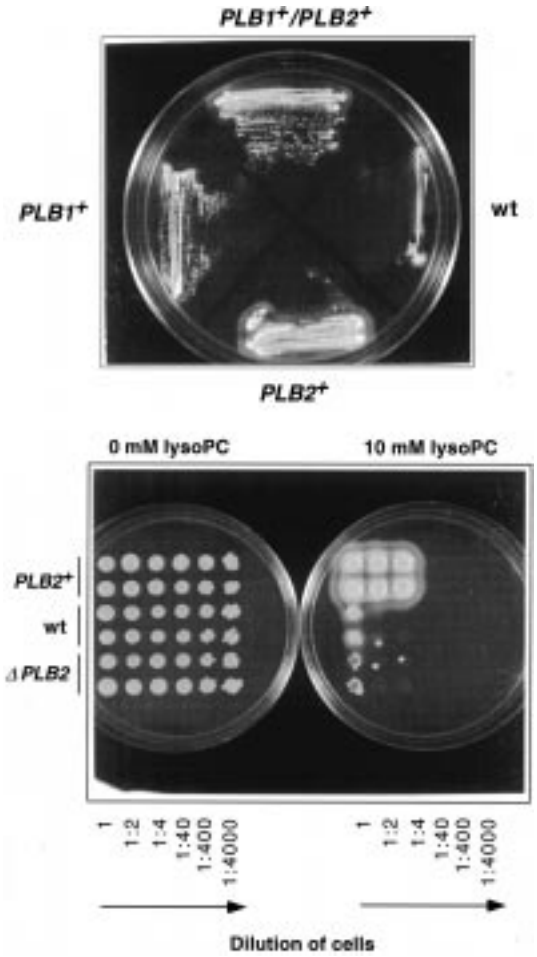

FIGURE 2: LysoPC resistance. Wild-type yeast and *PLB1*$^+$ and/or *PLB2*$^+$ overexpressing clones were inoculated onto YEPD plates containing 10 mM lysoPC (top panel). Cells were grown to saturation and an equal number of cells were collected and used for serial dilutions ranging from concentrated (1) to most dilute (1:4000). Cells were then transferred to plates containing 0 mM or 10 mM lysoPC (bottom panel).

Overexpression of *PLB1*, but not *PLB2*, resulted in increased transacylase activity, suggesting that *PLB2* is not capable of transferring fatty acyl groups between two lysoPC molecules. In support of this notion, we observed that deletion of *PLB1* caused complete loss of cellular transacylase activity, whereas deletion of *PLB2* was associated with enhanced transacylase activity (Table 2).

Another enzyme that could play an important role in yeast phospholipid remodeling and could possibly contribute to detoxification of lysoPC is LAT. This enzyme catalyzes the transfer of an acyl chain from acyl-CoA to lysoPC, synthesizing PC. To determine if LAT activity is present in yeast, the wild-type strain DG338 was incubated with [^{14}C]oleyl-CoA and lysoPC. We could detect the formation of labeled PC, indicating the presence of LAT activity (Table 2). To determine whether the products of either *PLB1* or *PLB2* encode an enzyme capable of performing this type of acyl transfer, LAT activity was measured in *PLB1*-overexpressing, *PLB2*-overexpressing, and Δ*plb1* and Δ*plb2* strains. All strains demonstrated comparable LAT activities, indicating that no such activity is encoded by the products of either *PLB1* or *PLB2*.

Phospholipid Analysis. To determine whether either deletion or overexpression of *PLB2* affected the phospholipid

Table 2: Enzyme Activities Associated with Wild-Type and *PLB1* and/or *PLB2* Deletion or Overexpression Strains[a]

	cell extract activity (nmol mg^{-1} min^{-1})				medium activity (nmol mL^{-1} min^{-1})
strain	phospholipase B	lysophospholipase	transacylase	LAT	lysophospholipase
wt	0.5 ± 0.2	96.6 ± 3.0	1.6 ± 0.2	9.1 ± 0.3	0.3 ± 0.1
Δ*plb1*	0.3 ± 0.1	19.3 ± 1.2	nd	9.2 ± 0.2	nd
Δ*plb2*	0.3 ± 0.2	18.2 ± 0.8	4.6 ± 0.4	9.7 ± 0.5	nd
Δ*plb1*/Δ*plb2*	nd	nd	nd	10.9 ± 0.7	nd
PLB2$^+$	5.3 ± 0.2	1012 ± 19.6	1.5 ± 0.3	8.4 ± 0.2	3.9 ± 0.1
PLB1$^+$	4.9 ± 0.6	948 ± 15.4	14.1 ± 0.6	8.2 ± 0.3	2.8 ± 0.4

[a] Strains were grown in rich medium to $OD_{600} = 2$. Cells were harvested and various enzyme activities were measured in both the cell extracts and the medium. Each assay performed with cell extract contained 10 μg of protein and incubation time was 10 min at 30 °C. Each assay performed with medium contained 100 μL of medium and incubation time was 1 h at 30 °C. Substrates used for the phospholipase B, lysophospholipase/transacylase, and LAT assays were 1,2-dipalmitoyl-PC, 1-palmitoyllysoPC, and 1-palmitoyllysoPC/oleyl-CoA, respectively. Results are the mean ± the standard deviation of four independent experiments. nd, is not detectable.

Table 3: Composition of the Major Phospholipid Classes in Wild-Type and *PLB1* and/or *PLB2* Deletion or Overexpression Strains[a]

	strain composition (nmol/g of wet cells)			
phospholipid classes	wt	Δ*plb2*	Δ*plb1*/Δ*plb2*	*PLB2*$^+$
PC	525.8 (51.2)	616.5 (54.1)	537.3 (53.8)	835.8 (56.4)
PE	270.8 (26.4)	310.8 (27.3)	186.8 (18.7)	419.0 (28.3)
PS	120.5 (11.7)	122.0 (10.7)	138.5 (13.9)	126.0 (8.5)
PI	109.0 (10.6)	89.8 (7.9)	136.5 (13.7)	101.8 (6.9)
total phospholipid	1026.1	1139.0	999.3	1482.5

[a] Strains were grown in rich medium and cells were harvested for phospholipid composition analysis. Results are average of two independent experiments. Numbers in parentheses represent percent of total phospholipid.

content or composition of yeast cells, both wild-type, *PLB2*-overexpressing, and Δ*plb2* strains were grown in rich medium, and lipid extractions were performed on samples harvested at log phase. Table 3 indicates that the distribution of the major phospholipids in the DG338 strain correlates well with previous studies (*17*) in that the phospholipid composition is approximately 51% PC, 26% phosphatidylethanolamine (PE), 12% phosphatidylserine (PS), and 11% phosphatidylinositol (PI). Neither deletion nor overexpression of *PLB2* had any appreciable effect on this distribution. However, the strain overexpressing *PLB2* showed a 1.5-fold increase in the amount of total phospholipid in the late growth phase.

Uptake of Fatty Acids and LysoPC and Distribution into Phospholipids. To gain more direct insight into the role of the *PLB2* gene product in lysoPC uptake and metabolism, and to determine the identity of the compound accumulating around the *PLB2*$^+$ colonies, wild-type and Δ*plb2* cultures were incubated with either [^{14}C]palmitate or 1-[^{14}C]palmitoyllysoPC (Table 4). Incubation of the strains with labeled palmitate revealed the following findings. First, the distribution of label into the major phospholipids was similar among all the strains. Second, the distribution of label correlated with the phospholipid composition listed in Table 3. However, a significant difference was observed when the strains were incubated with 1-[^{14}C]palmitoyllysoPC. In both the wild-type and the Δ*plb2* strains, the incorporation of label from lysoPC into PC was markedly increased in comparison to incorporation of label from fatty acid into PC. In contrast, the incorporation of label from lysoPC into PE, PS, and PI in these strains markedly decreased in comparison to incorporation of label from fatty acid. In these strains, as much as 85−90% of the label was found in PC. This effect was not observed in the *PLB2*-overexpressing strain, in which significantly more label was found associated with PE, PS, and PI. Together, these results suggest that the observed

Table 4: Lipid Uptake and Metabolism of Wild-Type and *PLB2* Deletion or Overexpression Strains[a]

	phospholipid classes (% of total counts)		
strain	PC	PE	PS/PI
	[^{14}C]Palmitate		
wt	64.1 ± 7.6	20.4 ± 4.3	15.6 ± 5.1
Δ*plb2*	65.8 ± 8.5	22.3 ± 4.1	11.92± 3.2
PLB2$^+$	72.6 ± 5.1	17.1 ± 2.3	10.4 ± 2.6
	[^{14}C]Palmitoyllyso-PC		
wt	85.6 ± 3.2	7.9 ± 1.1	6.5 ± 1.5
Δplb2	90.7 ± 4.1	4.8 ± 0.9	4.5 ± 1.4
PLB2$^+$	75.9 ± 6.9	11.5 ± 2.9	12.6 ± 3.2

[a] Strains were incubated for 3 h at 30 °C in minimal medium containing either 50 μM [^{14}C]palmitate (**a**) or [^{14}C]palmitoyl-lysoPC (**b**). Lipids were extracted from the cells and the phospholipids were analyzed for ^{14}C label. Results are the mean ± the standard deviation of three independent experiments.

increase in labeled PC (from lysoPC as compared to fatty acid) in both the wild-type and the Δ*plb2* strains is a result of cellular uptake of lysoPC followed by its acylation to form PC. In the *PLB2*, overexpressing strain, the vast majority of the lysoPC is hydrolyzed, releasing free fatty acid and leaving very little lysoPC for direct reacylation. The free fatty acids liberated in this strain can then be activated and incorporated into other phospholipids, such as PI, PS, and PE. In support of this explanation, when the medium from the *PLB2*-overexpressing strain was analyzed, 90% of the lysoPC was found to be hydrolyzed to the corresponding fatty acid and glycerophosphocholine, suggesting that the material deposited around *PLB2*$^+$ colonies grown on lysoPC agar plates could be fatty acids.

Effects of PLB1 and PLB2 Deletions on Growth. Next, we examined the effect of *PLB2* expression on yeast growth under various conditions. Whereas no difference in growth between wild-type and Δ*plb2* strains was noted under rich conditions at 30 °C, we found that the Δ*plb1* strain and the

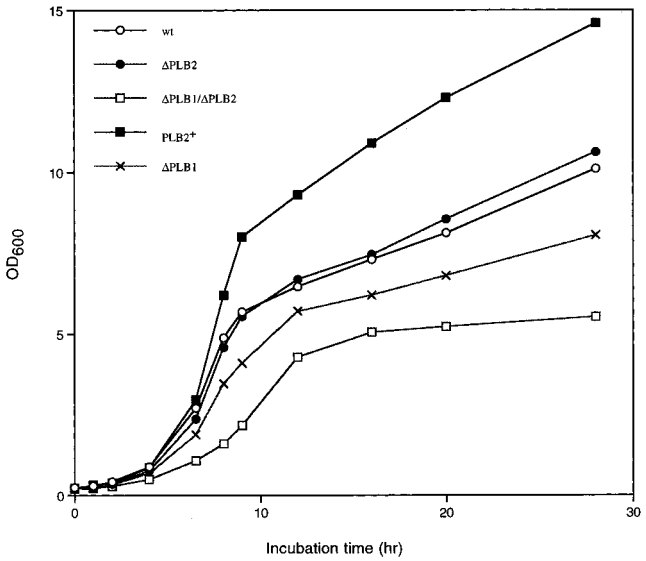

FIGURE 3: Yeast growth curves. Yeast strains were grown to exponential phase and reinoculated into rich medium at 30 °C.

Δ*plb1*/Δ*plb2* strain grew more slowly than wild type, with the double deletion strain growing considerably slower than the single Δ*plb1* strain (Figure 3). This suggests that *PLB1* and *PLB2* are each able to provide a threshold amount of lysophospholipase activity and/or to compensate to some degree for the loss of the other gene product, whereas total loss of lysophospholipase activity greatly inhibits the growth of the double deletion strain. In contrast, the *PLB2*-overexpressing strain could reach a higher cell density before saturation than all the other strains.

To determine the role of *PLB2* in confirming resistance to lysoPC, wild-type, Δ*plb2*, Δ*plb1*/Δ*plb2*, and *PLB2*-overexpressing strains were grown in minimal medium supplemented with lysoPC. The *PLB2*-overexpressing strain was only slightly affected by 200 μM lysoPC, whereas the growth of both the wild-type and Δ*plb2* strains was severely inhibited under this condition. The inhibitory effect of lysoPC was most noticeable in the double deletion strain, which demonstrated sensitivity to as little as 50 μM lysoPC, a concentration that had no effect on the growth of wild-type or Δ*plb2* strains. Aside from lysoPC sensitivity, no differences in morphology or other phenotypes distinguished the Δ*plb2* strain from the wild type (including growth on glycerol, at 14 °C, at 37° C, or under high osmolarity).

Biochemical Characterization of Plb2p. To characterize Plb2p, a plasmid containing the *PLB2* gene under control of the *GAL1* promoter was created and introduced into the double deletion strain Δ*plb1*/Δ*plb2*. Transformants were selected for uracil prototrophy and then were grown in YP medium containing galactose to an $OD_{600} = 2$. Plb2p was released from the periplasmic space following treatment of the cells with zymolyase, and lysophospholipase activity of the released Plb2p was measured by adding different lysophospholipids at pH 4 (Table 5). The preferred substrate for Plb2p under these conditions was lysoPC. In addition to lysoPC, both lysoPS and lysoPE were hydrolyzed by the enzyme. No hydrolysis was found when either lysoPI or lysoPA was used as substrate. The periplasmic extract of the double deletion strain Δ*plb1*/Δ*plb2* had no lysophospholipase activity toward any of the lysophospholipids added under the conditions used. The *Plb2*-associated lysophospholipase activity toward lysoPC showed a pH optimum of 4 (Figure 4) and a temperature optimum of 40 °C (Figure 5).

Table 5: Lysophospholipase Activity of PLB2p[a]

substrate	Plb2p activity (μmol mg^{-1} min^{-1})
1-palmitoyllysoPC	3.26 ± 0.35
1-palmitoyllysoPS	1.46 ± 0.14
1-palmitoyllysoPE	0.90 ± 0.02
1-palmitoyllysoPA	nd
bovine liver lysoPI	nd

[a] The *PLB2* gene under control of the *GAL1* promoter was introduced into the double deletion strain Δ*plb1*/Δ*plb2* and the culture was grown in rich medium containing galactose to $OD_{600} = 2$. Cells were harvested and a periplasmic extract was prepared by zymolyase treatment. Each assay performed with the periplasmic extract contained 5 μg of protein and incubation time was 5 min at 30 °C. Results are the mean ± the standard deviation of three independent experiments. nd, not detectable. A periplasmic extract of the double deletion strain Δ*plb1*/Δ*plb2* showed no residual lysophosphospholipase activity toward any of the lysophospholipid substrates under the conditions used.

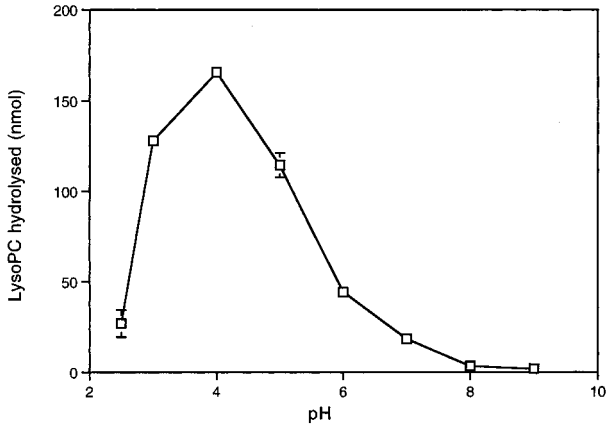

FIGURE 4: pH optimum of the Plb2p-associated lysophospholipase activity. Each assay contained 5 μL of periplasmic extract and 270 nmol of lysoPC in a total of 500 μL of buffer. Samples were incubated at 37 °C for 10 min. The buffers used were glycine hydrochloride (pH 2−3), glycine acetate (pH 4−6), and Tris-HCl (pH 7−9) in concentrations of 100 mM.

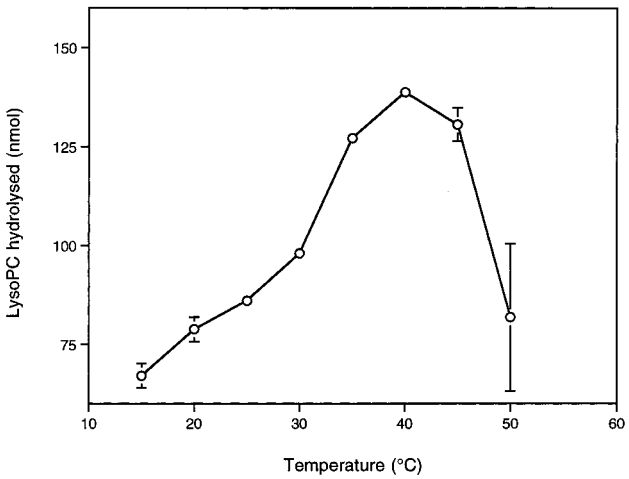

FIGURE 5: Temperature optimum of the Plb2p-associated lysophospholipase activity. Each assay contained 5 μL of periplasmic extract and 270 nmol of lysoPC in 500 μL of 100 mM glycine acetate, pH 4. Samples were incubated for 10 min.

Transcriptional Regulation of PLB2. To determine whether *PLB2* expression is affected by nutrient conditions, we

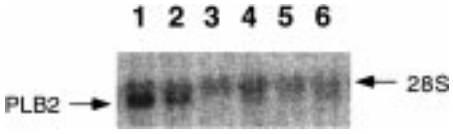

FIGURE 6: *PLB2* expression is transcriptionally regulated. Lanes 1−6 represent total RNA obtained from wild-type yeast cells grown in YEPD to $OD_{600} = 2$, 5, 5.8, 6, 6.6, and 7.5, respectively. 28S represents the 28S ribosomal RNA band. PLB2 represents *PLB2* message.

evaluated the abundance of *PLB2* transcript in a wild-type strain at different points throughout the growth curve. To ensure that only the *PLB2* gene transcript would be identified, a region of the *PLB2* open reading frame between nucleotides 1759 and 2061 was amplified by PCR and utilized as a probe. This region displays homology neither to the *PLB1* open reading frame nor to the open reading frame YOL011w, which is also homologous to *PLB1*. As can be seen in Figure 6, *PLB2* message was abundant during exponential-phase growth conditions but was markedly reduced after cultures attained a density of $OD_{600nm} = 3$ or greater, indicating that Plb2p expression is regulated at the transcriptional level and that transcription is maximal during log-phase growth. The specific factors that influence *PLB2* transcription (phospholipids, inositol, carbon source, or possibly other enzyme activities) remain to be elucidated.

DISCUSSION

In this study we used a genetic screen to isolate high-copy suppressors of lysoPC toxicity. This approach has led to the identification of *PLB1* as a lysoPC resistance gene and the elucidation of the functional identity of *S. cerevisiae* open reading frame YMR006c. This gene, which we refer to as *PLB2*, is 64% identical to *PLB1* and, similar to *PLB1*, encodes a protein that demonstrates phospholipase B/lysophospholipase activities toward PC and lysoPC. However, unlike *PLB1*, this gene does not encode a transacylase activity. A database search did not reveal specific conserved residues between Plb1p and other transacylase genes. Molecular modeling in combination with site-directed mutagenesis may be required to determine potential amino acid residues involved in the transfer of acyl groups between two lysoPC molecules.

Whereas disruption of either *PLB1* or *PLB2* open reading frames by homologous recombination abolished 80% of total cellular phospholipase B/lysophospholipase activities, disruption of both genes in the same strain was associated with complete loss of phospholipase B/lysophospholipase activities assayed with PC and lysoPC substrates at pH 4. These findings suggest that the contributions of *PLB1* and *PLB2* to total cellular phospholipase B/lysophospholipase activities are not simply additive and may reflect some interdependence of these two enzymes. Plb2p obtained from the periplasmic space hydrolyzed lysoPC, lysoPS, and lysoPE but was unable to hydrolyze lysoPI and lysoPA when enzyme activity was measured at pH 4.0. The periplasmic extract of the double deletion strain Δ*plb1*/Δ*plb2* had no detectable lysophospholipase activity toward any of the lysophospholipids used as substrates. These results suggest the presence of an uncharacterized lysophospholipase activity in *S. cerevisiae* capable of hydrolyzing lysoPI and lysoPA. It is possible that Plb1p, which has been shown to hydrolyze lysoPC, can hydrolyze other lysophospholipid substrates as well. Moreover, searching the *Saccharomyces* database with the *PLB1* and *PLB2* sequences revealed a third potential lysophospholipase gene on chromosome XV, designated by the open reading frame YOL011w, which demonstrates more than 60% homology to the other *PLB* genes. Additional experiments performed with different deletion and overexpressing strains could reveal an interesting and very important relationship between these enzymes in regulating levels of lysophospholipids in *S. cerevisiae*.

We speculate that both Plb1p and Plb2p function in the regulation and remodeling of membrane phospholipid classes. In support of this, we observed that labeled lysoPC had different fates when incubated with the different strains. For example, in the *PLB2*$^+$ strain, lysoPC was hydrolyzed and the resulting free fatty acid was presumably activated and then incorporated into all the phospholipid classes examined, facilitating phospholipid remodeling, whereas in the wild-type and phospholipase deletion strains, a substantial portion of the exogenous lysoPC could not be hydrolyzed and was incorporated into membranes and subsequently reacylated to PC.

The analysis of phospholipid composition of wild-type, *PLB2*$^+$, and deletion strain membranes did not reveal substantial differences among the strains under standard conditions in rich medium. These findings likely reflect the rigidly regulated phospholipid composition of yeast cell membranes under these conditions. However, we have not examined the phospholipid composition of the strains under other conditions, where remodeling is likely to occur. We cannot, therefore, discount the possibility that deletion of phospholipase B/lysophospholipases may have an effect on membrane responses to stress. In contrast, the finding that the *PLB2*$^+$ strain demonstrated a higher total phospholipid content than the other strains suggests that total phospholipid content is not rigidly maintained and may be influenced by the amount of fatty acid resources, made available by abundant phospholipase B/lysophospholipase activity.

In rich medium the Δ*plb1*/Δ*plb2* strain grew considerably more slowly than the wild type, whereas the growth rate of the *PLB2*$^+$ strain was faster, and saturation was attained at a higher culture density. The differences in growth rate may reflect the different abilities of these strains to utilize available lipid substrate as fuel. However, other indirect effects on proliferation, the general well-being of the cell, and/or its ability to tolerate stress that may result as a consequence of phospholipase B/lysophospholipase expression cannot be discounted.

Northern analysis of the wild-type strain indicates that *PLB2* transcription is maximal during log-phase growth, and the abundance of message diminished significantly after cultures reached $OD_{600} = 3$. The activities of numerous enzymes required for phospholipid metabolism in yeast are affected by growth phase and the availability of carbon source, inositol, and nitrogen (*12*, *48*). This is mediated, at least in part, through coordinated transcription regulation of phospholipid biosynthetic genes (*11*, *49*−*51*). The transcriptional downregulation of *PLB2* might also explain the growth advantages displayed by the *PLB2*$^+$ strain, in which the levels of this enzyme are artificially elevated and are not likely to be subject to the normal regulatory mechanisms operative within the cell. If phospholipase B/lysophospholipase activity

is normally low in late-phase growth, then unregulated expression and secretion of phospholipase B/lysophospholipase might enhance utilization of extracellular complex lipids when all other carbon sources have been depleted in the medium, allowing some additional growth.

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