Characterization and Function in Vivo of Two Novel Phospholipases B/Lyso phospholipases from Saccharomyces cerevisiae*

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The yeast genome contains two genes, designated as PLB2 and PLB3, that are 67% and 62% identical, respectively, to PLB1, which codes for a phospholipase B/lyso phospholipase in yeast (Lee, S. K., Patton, J. L., Fido, M., Hines, L. K., Kohlwein, S. D., Paltauf, F., Henry, S. A., and Levin, D. E. (1994) J. Biol. Chem. 269, 19725–19730). Deletion and overexpression studies and in vivo and in vitro activity measurements suggest that both genes indeed code for phospholipases B/lyso phospholipases. In cell free extracts of a plb1 plb2 plb3 triple mutant, no phospholipase B activity was detectable. Upon overexpression of PLB2 in a plb1 plb3 mutant background, phospholipase B activity was detectable in the plasma membrane, periplasmic space extracts and the culture supernatant. Similar to Plb1p, Plb2p appears to accept all major phospholipid classes, with a preference for acidic phospholipids including phosphatidylinositol 3’,4’-bisphosphate and phosphatidic acid. Consistent with a function as an extracellular lyso phospholipase, PLB2 overexpression conferred resistance to lyso phosphatidylcholine. Deletion of Plb2p function had no effect on glycerophosphoinositol or glycerophosphocholine release in vivo, in contrast to a deletion of Plb3p function, which resulted in a 50% reduction of phosphatidylinositol breakdown and glycerophosphoinositol release from the cells. In vitro, Plb3p hydrolyzes only phosphatidylinositol and phosphatidylserine and, to a lesser extent, their lysol analogs. Plb3p activity in a plb1 plb2 mutant background was observed in periplasmic space extracts. Both Plb3p and Plb2p display transacylase activity in vitro, in the presence or absence, respectively, of detergent.

Phospholipases B catalyze the hydrolytic cleavage of both acylster bonds of glycerophospholipids. Products of phospholipase B activity are fatty acids and water-soluble glycerophosphodiesters. In yeast, soluble degradation products are released into the culture medium, and are thus an indicator of cellular phospholipase B activity. The only acylster-hydrolyzing enzyme from yeast characterized so far at the molecular level is a phospholipase B encoded by the PLB1 gene (1). The highly glycosylated enzyme of about 220 kDa (73 kDa for the protein part, predicted from the sequence) is enriched in the yeast plasma membrane but was also found in the periplasmic space and in the culture supernatant. The lyso phospholipase activity of Plb1p greatly exceeds the activity catalyzing the first step of hydrolysis; thus, lyso phospholipids do not accumulate as intermediate products of Plb1p activity (2–4). In addition, this enzyme has transacylase activity, catalyzing the synthesis of phosphatidylcholine (PtdCho)1 from two molecules of lyso phosphatidylcholine. The physiological function of yeast phospholipase B is still unclear; neither disruption of the PLB1 gene nor its overexpression result in detectable growth phenotypes. However, the amount of glycerophosphocholine (GroPCho) and glycerophosphoethanolamine released into the culture supernatant by a plb1 deletion mutant is greatly reduced compared with wild type. In contrast, release of glycerophosphoinositol (GroPIns) is hardly affected in this strain, suggesting that Plb1p is mainly responsible for the deacylation of PtdCho and phosphatidylethanolamine (PtdEtn), but not phosphatidylinositol (PtdIns). Thus, additional enzyme(s) must exist which catalyze deacylation of PtdIns (1, 5).

A search in the yeast genome data base revealed the existence of two open reading frames with significant homology to PLB1. Here we report on the cloning and molecular characterization of these genes, designated as PLB2 and PLB3, and the characterization of enzyme activities in vivo and in vitro resulting from deletion or overexpression of these genes.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Yeast strains used in this study (Table I) were derivatives of W303 (R. Rothstein). Cultures were grown on YEPD medium (1% yeast extract (Difco), 2% peptone (Difco), 2% glucose). Synthetic minimal medium (7) supplemented with the appropriate nutrients was used for plasmid maintenance. YEPD media plates supplemented with 0.01% Geneticin (G418, Fluka) were used for selection of transformants in gene displacement experiments (8). For growth inhibition studies, YEPD media were supplemented with up to 50 μg/ml lyso-PtdCho and cells were incubated for 18 h. Culture media used for measuring the release of glycerophosphoinositol and glycerophosphocholine in vivo (5) were supplemented with 100 μM each of choline, glycerophosphocholine, inositol, and glycerophosphoinositol. For anaerobic growth tests, yeast strains were precultivated overnight in synthetic minimal medium (7). 0.5 ml of cell culture were harvested and washed twice to remove residual medium. Cells were resuspended in sterile water to an optical density of 1. A 3-μl aliquot of cell suspension and of 10−2, 10−3, and 10−4 dilutions, respectively, were dropped onto agar plates containing Brij 58 (1%), Ergosterol (0.002%), and either Tween 80 (0.5%) or palmitoyl-oleoyl-phosphatidylcholine (0.12%). Agar plates were incubated in an anaerobic jar in the presence of AnaeroGen (Oxoid) for 4 days.

Miscellaneous Techniques—Yeast was transformed by the lithium

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† The abbreviations used are: PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; GroPIns, glycerophosphoinositol; GroPCho, glycerophosphocholine; GPI anchor, glycosylphosphatidylinositol anchor; PtdIns-bisP, phosphatidylinositol-3’,4’-bisphosphate; PL, phospholipid; bp, base pair(s); PCR, polymerase chain reaction; WT, wild type.

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acetate method (9). Genetic manipulation, yeast DNA isolation, and DNA sequencing were carried out as described (10). Computational sequence analysis was performed using PILEUP from EGGCG Wisconsin package version 8.1.0 (1996). *Saccharomyces* Genome Database, Yeast Proteome Database, and the most recent data bases from GenBank and SwissProt available via the World Wide Web.

### Disruption of PLB3

The open reading frame YMR006c, designated PLB2, was replaced by the *kanMX* gene in a PCR-based gene disruption experiment using the long flanking sequence homology protocol (8). The following sets of primers were used: P5’ (5’-GGGATCCTGATCC-3’ and the hybrid primer P5’L (5’-GGGATCGTTCGACCTGCAGCGTACGACTTTATTCCTCTTTTCCAA-176), and P3’ (5’-CGGAAAGGGATCCTGATCC-3’)) to amplify a 254-bp fragment at the 5’ region of PLB2 (–78 to +176), and P5’L (5’-AAGGAGCTCTCAAGATG-3’) and the hybrid primer P5’L (5’-AAGGAGCTCTCAAGATG-3’)) to amplify a 329-bp fragment at the 5’ end of PLB2 (+1913 to 120 bp downstream of the stop codon). Yeast genomic DNA was used as template for the PCR reactions (Es *Toq* polymerase, TaKaRa). The fragments were used as primers for a second PCR, to amplify the KanMX-disruption cassette (2289 bp length) as template. PCR products were transformed into yeast (see below).

**Disruption of PLB3**—The open reading frame YLO11w, designated PLB3, was replaced by the *kanMX* gene in analogy to the PLB2 disruption described above. The following sets of primers were used: P5’ (5’-TTAGATGCTGGAGGATCC-3’ and the hybrid primer P5’L (5’-GGGATCCTGATCC-3’)) and the hybrid primer P5’L (5’-GGGATCCTGATCC-3’)) to amplify 434 bp of the 5’ region of PLB3 (+233 to +201 and 5’-AACGAGCTCGAATTCATCGATGATAGGATACTACCCCAT-AC-3’)) to amplify 346 bp of the 3’ end of PLB3 (+1773 to 57 bp downstream of the stop codon). Yeast genomic DNA was used as template for the PCR reaction, and the obtained fragments were used as primers for a second PCR to amplify the KanMX-disruption cassette (2298 bp length). PCR products were transformed into yeast (see below).

Diploid wild type and a heterozygous plb1Δura3/PLB1 plb2Δura3/PLB2 mutant strain were transformed with the PCR products and selected for growth on YDO medium containing Geneticin (8). Resistant colonies were stored and incubated for 3 days. Correct integration of the disruption cassette was confirmed by DNA sequencing.

**Cloning of PLB2 and PLB3 into the Episomal Plasmid YEp351**—The PLB2 gene including 908 bp upstream of the putative translation start site and 211 bp downstream of the stop codon, was amplified by PCR from total yeast genomic DNA using the following set of primers: primer A (5’-GTTCTAGACATATTGATGGCCGGTACAC-3’) and primer B (5’-GTTCTAGACATATTGATGGCCGGTACAC-3’). The PCR product (3189 bp) was purified, cut with XbaI, and ligated into the XbaI site of plasmid pYEp351 (12). Sequence of the PCR-cloned PLB2 gene was confirmed by DNA sequencing.

### Phospholipase Activity in Vivo: Release of Glycero phospho[14C]Choline and Glycero phospho[H3]Inositol into the Medium

**Preparation of Periplasmic Extracts**—Cells were grown in choline- and inositol-free media to early logarithmic phase (OD600 of 3). Cells were harvested and washed once with ice-cold water and buffer A (1.2 M sorbitol, 10 mM KH2PO4, pH 7.4). Cells were resuspended in buffer A containing 3 mg/ml zymolyase 20T (Seikagaku) to a density of 0.3 g of wet cells/ml and incubated under gentle agitation for 30 min at 30 °C. Cell wall digestion was monitored by microscopy. Spheroplasts were removed by centrifugation (GSA rotor, 700 × g, 20 min), and the supernatant was collected for enzyme activity measurements.

### Preparation of Radioactively Labeled Phospholipid Substrates—Radioactively labeled phospholipids were prepared biosynthetically by incubating yeast cells with [1-14C]palmitic acid (NEN Life Science Products) for 4 h, followed by extraction of lipids and isolation of phospholipid classes by thin-layer chromatography (16). Purity was typically >95%, as checked by TLC. Radioactively labeled lyso-phospholipids were prepared from [1-14C]palmitate-labeled phospholipids by hydrolysis with phospholipase A2 from *Naja naja* (Sigma) and TLC separation (16). Radioactively labeled phospholipid and detergent dissolved in chloroform/methanol (2:1, v/v), evaporation of the solvent under a stream of nitrogen, and subsequent dispersion in water (17).

### Assay of Phospholipase Activity—Enzyme activity was determined in an assay system containing 50 mM glycine/HCl, pH 3.5, or 50 mM citrate buffer, pH 5.5, radioactively labeled phospholipids with a specific radioactivity of about 0.4 mCi/mmol, and, if not indicated otherwise,
periplasmic space extracts equivalent to 13 mg of wet cells as the enzyme source, in a total assay volume of 100 μl. Substrate was added in the form of phospholipid/Triton X-100 mixed micelles at ratios specified in the respective figures and tables. Concentration of Ca²⁺ was as indicated in the figure legends. The reactions were started by the addition of substrate, and incubation was continued at 30 °C for 10 min, which is in the linear range of the reaction. The reaction was stopped by the addition of 4 volumes of chloroform/methanol (2:1, v/v). For complete extraction of lyso-PtdIns, the reaction mixture was acidified with 3 mol of 1 N HCl. Aliquots of the organic phase were applied to TLC plates, and lipids were separated with chloroform/methanol/6% ammonia (60:40:5, v/v/v) as the developing solvent. Radioactivity of individual lipids was quantified using an automatic TLC linear analyzer (Trace-master 20, Berthold), or by liquid scintillation counting.

Km and v max values were determined by non-linear regression to the Michaelis-Menten function using the computer program SigmaPlot 4.0.1.

Lipid and Protein Analyses—Lipids were extracted from cells after homogenization with glass beads in a Braun cell homogenizer (Braun, Melsungen, Germany), with chloroform/methanol (2:1, v/v) as described (18). Individual phospholipid classes were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% ammonia (65:35:5, v/v/v) as the developing solvent. Radioactivity of individual lipids was quantified using an automatic TLC linear analyzer (Trace-master 20, Berthold), or by liquid scintillation counting. Km and v max values were determined by non-linear regression to the Michaelis-Menten function using the computer program SigmaPlot 4.0.1.

RESULTS

Characterization of PLB2 and PLB3 Genes—The yeast genome contains two genes, designated as PLB2 and PLB3, with high homologies to PLB1 that was previously shown to code for a phospholipase B/lysophospholipase in yeast (1). PLB2 is located 1 kilobase pair downstream of PLB1 on chromosome XIII and is 66.8% identical at the DNA level and 73.5% identical at the protein level to Plb1p (1). PLB2 codes for a 706-amino acid protein of 75.4 kDa. Several putative N- and O-glycosylation sites are present. In contrast to Plb1p (1) and Plb3p (see below), the protein encoded by PLB2 does not appear to contain a cleavable N-terminal signal sequence nor does the C terminus contain the typical motifs for GPI anchor attachment (21–24).

The PLB3 gene, located on chromosome XV, is 62.0% identical to PLB1 at the DNA level and 73.4% identical at the protein level (Fig. 1). Plb2p and Plb3p are 66.0% identical. The PLB3 gene codes for a 686-amino acid protein of 75.0 kDa. The N and C termini harbor stretches of 18 and 16 hydrophobic amino acids, respectively, indicative of membrane spanning domains (Fig. 1). The N terminus may contain a cleavable signal sequence, with a predicted cleavage site between amino acids 20 and 21 (21). Adjacent to the C-terminal TM domain a motif for GPI anchor attachment on Asn-659, similar to Plb1p, is present (Fig. 1) (22). PSORTII (23) predicts a high probability for a localization of Plb3p in the plasma membrane and in the extracellular space, similar to Plb1p, which is consistent with findings of Hamada et al. (24).
evidence that Plb3p is localized in the extracellular space. Plb2p is predicted to localize to the extracellular space as well, and with a low probability to mitochondria.

Homology of PLB2 and PLB3 extends not only to Saccharomyces cerevisiae PLB1 (67% and 62%, respectively) but to structural genes coding for phospholipases B in Schizosaccharomyces pombe (29% and 30%; GenBank accession no. AB005603), Penicillium notatum (43% and 45%; see Ref. 25), Candida albicans (51% and 52%; see Ref. 26), and Torulaspora delbrueckii (63% and 53%; see Ref. 27). The high homology to established phospholipase B encoding genes further suggests that PLB2 and PLB3 are indeed structural genes coding for phospholipases B/lyso-phospholipases in S. cerevisiae.

Phenotypic Analysis of plb2 and plb3 Deletion Strains and of Transformants Overexpressing PLB2 or PLB3—In order to further characterize the PLB2 and PLB3 genes and to verify their role as structural genes for phospholipases, deletion mutants and strains overexpressing the respective genes were constructed. plb2 mutants and plb1 plb2 double mutants grew at slightly reduced rates compared with wild type cells on complete YEPD media, whereas plb3 mutants and plb1 plb3 double mutants and a plb1 plb2 plb3 triple mutant grew at wild type rates on fermentable and non-fermentable carbon sources, at temperatures between 25 °C and 37 °C. Sporulation efficiencies of heterozygous diploid plb2/PLB2 and plb3/PLB3, and homozygous diploid plb2/plb2 or plb3/plb3 mutants were comparable to wild type; sporulation and mating efficiency were also not affected by any of the double or triple mutations. Overexpression of PLB2 or PLB3 from a high copy number plasmid had no apparent effect on cellular growth under the conditions tested. Upon incubation of cells in water overexpression of PLB2 and PLB1, but not of PLB3, resulted in accelerated cell death; after 1 week of incubation of PLB2- or PLB1-overexpressing strains in distilled water, only 30% of the cells survived, whereas no loss of viability was observed with WT and PLB3-overexpressing strains under these conditions.

Utilization of Exogenous Phospholipids—The ether analog of lyso-phosphatidylcholine, 1-O-hexadecyl-sn-glycero-3-phosphocholine, is toxic to wild type yeast cells (28). We assumed that lyso-PtdCho might be equally toxic unless it was effectively metabolized prior to exerting its toxic effects, e.g. by an extracellular lyso-phospholipase. Thus, we tested the effect of deletion and overexpression of PLB2 and PLB3 on lyso-PtdCho toxicity. plb2 mutants were more sensitive than the wild type strain and growth of a plb1 plb2 plb3 triple mutant was essentially abolished at 10 µg/ml lyso-PtdCho in the growth medium. Overexpression of PLB2 increased resistance against lyso-PtdCho but not against the non-hydrolyzable ether analog (Edelfosine; data not shown), suggesting that the protective function of Plb2p results from its lysophospholipase activity (Fig. 2; see also in vitro experiments shown below, Table III). Sensitivity of cells toward lyso-PtdCho was not affected by either deletion or overexpression of PLB3, consistent with the inability of Plb3p to hydrolyze lyso-PtdCho (Table III, see below).

When yeast cells are grown under anaerobic conditions, they are dependent on an external source of ergosterol and unsaturated fatty acids. When the sole source of fatty acid in the growth medium is phosphatidylcholine, external phospholipases B could play a role in phospholipid digestion. On media containing Tween 80, which provides a source of unsaturated fatty acids, wild type and a plb triple disruption strain (MF17) grew at the same rate (Fig. 3A). In contrast and consistent with a function of phospholipase B in extracellular phospholipid breakdown, growth of a plb triple disruption strain on a medium containing palmitoyl-oleoyl-phosphatidylcholine as the only source for unsaturated fatty acids under anaerobic conditions was severely impaired compared with wild type cells (Fig. 3B).

Phospholipid Metabolism in plb Mutants—The total cellular phospholipid content of plb1 plb2 plb3 triple mutant cells was significantly increased (3.6 ± 0.3% of cell dry weight) as compared with wild type cells (2.6 ± 0.2% of cell dry weight); however, their phospholipid composition was not significantly altered (data not shown). Triacylglycerol levels of the triple mutant were reduced by 50% compared with wild type cells. This observation indicates a role of phospholipases B in fatty acid distribution between membrane phospholipids and fatty acid esters in the form of triacylglycerols.

Phospholipid Degradation in Vivo and Release of Glycerophosphodiester from plb1, plb2, and plb3 Mutant Cells—Cellular phospholipase B activity can be monitored by the release of soluble phospholipid degradation products into the growth medium (1, 5). Previous studies have shown that deletion of the PLB1 gene resulted in a significant reduction in GroPCho release from the cells, whereas the production of GroPIns was hardly affected (1). As summarized in Table II, deletion of PLB3 reduced GroPIns release by approximately 50%, whereas GroPChe release was elevated by 60% in the plb3 mutant, compared with the wild type control. Preliminary experiments
suggested an increase in PLB1 expression in cells deficient in Plb3p, indicating cross-regulation between yeast phospholipases B (data not shown). Deletion of PLB2 had no effect on GroPCo or GroPlns release. Mutants deficient in all three PLB genes showed residual release of GroPCo and GroPlns below 10% of wild type. Overexpression of PLB3 from a multicopy plasmid in plb1 plb2-deficient cells restored GroPlns, but not GroPCo release, whereas overexpression of PLB2 had no effect on GroPlns or GroPCo secretion (Table II).

**Table II**

| Strain (relevant genotype) | Glyerophosphodiester release after 6 h | |
|---------------------------|---------------------------------------|---|
|                           | GroPlns     | GroPCo  |
| WT                        | 5           | 8       |
| MF17 (plb1 plb2 plb3)     | <1          | <1      |
| MF3 (plb1)                | 5           | <1      |
| MF11 (plb2)               | 6           | 7       |
| MF12 (plb3)               | 2           | 13      |
| MF28 (plb1 plb2 plb3 [YEp{PLB2}]) | <1   | <1      |
| MF29 (plb1 plb2 plb3 [YEp{PLB3}]) | 7       | <1      |

Detection of phospholipase B activity in periplasmic space extracts required the presence of detergents. In order to characterize enzyme activities resulting from expression of only PLB1, PLB2, or PLB3, the respective genes were overexpressed in a plb1 plb2 plb3 triple deletion mutant harboring either PLB1, PLB2, or PLB3 on a multicopy plasmid. No phospholipase B activity was detectable in cell-free preparations from the untransformed triple mutant strain using Triton X-100 as the detergent, and lysophospholipase activity was reduced to below 10% of the wild type control.

With phospholipid/Triton X-100 mixed micelles, optimum activity for all three enzymes was observed at pH 2.5–3.5 and no activity was detectable at pH 5.5–7 with this detergent.

In order to characterize substrate specificities in more detail, we have determined enzyme activities either with a constant molar ratio of phospholipid and Triton X-100 or, in a second set of experiments, we varied the molar fraction of phospholipids in the range from 0 to 0.16 at a constant detergent concentration of 40 mM Triton X-100. Kinetic data obtained with a constant molar ratio of phospholipid to detergent yielded hyperbolic curves which fit the Michaelis-Menten equation (Fig. 4A). In contrast, varying phospholipid-detergent molar ratios resulted in a sigmoidal relation between reaction rates and substrate concentration (Fig. 4B), which is consistent with a kinetic model for phospholipase A activity described previously (30). However, both experimental designs showed the same apparent substrate preference of the respective enzymes.

**Enzymatic Properties of PLB1**—Kinetic data on the substrate specificity as obtained at pH 3.5 using Triton X-100 mixed micelles are summarized in Table III. Highest catalytic efficiency of PLB1 was observed with PtdSer as the substrate, followed by PtdIns and (at about 1000-fold lower efficiency) with PtdCho and PtdEtn.

**Enzymatic Properties of PLB2**—When assayed under acidic conditions (pH 3.5), periplasmic extracts of PLB2-overexpressing cells had high phospholipase B activity toward the major phospholipid classes in the order PtdSer > PtdIns > PtdCho > PtdEtn (Table III). Specific activity based on cell weight was about 10-fold lower compared with PLB1 specific activity toward the acidic phospholipid substrates, but in the same range for PtdEtn and PtdCho. Lysophospholipase activity exceeded phospholipase B activity by a factor of 70 in the case of choline phospholipids (Table III). Phosphatidic acid and PtdIns-bisP were also utilized as substrates but with lower efficiencies than the other acidic phospholipids (data not shown). During incubation in the presence of lysophospholipids, but only in the absence or at very low (<0.4 mM) concentrations of Triton X-100, we observed accumulation of diacylphospholipids, indicating that the enzyme has transacylase activity that is inhibited by the detergent (data not shown). Phospholipase B and lysophospholipase activities in extracts from cells expressing PLB2 were independent of Ca²⁺ at pH 3.5.
Enzymatic Properties of Plb3p—Phospholipase B activity resulting from overexpression of PLB3 was selective for PtdIns and PtdSer, whereas PtdCho and PtdEtn were not cleaved by Plb3p in vitro (Table III). In addition, phosphatidic acid and PtdIns-bisP were not cleaved by Plb3p. Incubation of acyl-labeled PtdIns with periplasmic extract from PLB3-expressing cells resulted in the formation of lyso-PtdIns, besides an excess of labeled fatty acids (Fig. 5B). Thus, Plb3p appears to be a phospholipase B with rather low lysophospholipase activity, which was also confirmed by direct measurement of lysophospholipase activity. Accumulation of PtdIns during incubation with lys-PtdIns at pH 3.5 demonstrates that the enzyme has transacylase activity (Fig. 5C). With PtdIns as the substrate, enzyme activity was stimulated by 5 mM Ca²⁺, similar to the PtdIns-selective phospholipase B from S. pombe (29). Lysophospholipase activity toward lyso-PtdIns and lyso-PtdSer was also stimulated by Ca²⁺.

The high preference of Plb2p and Plb3p for negatively charged phospholipids as substrates at pH 3.5 is striking. Therefore, we tested whether it was true substrate specificity or preference for a negatively charged surface of substrate micelles, by using mixed substrates in the enzyme assay. In mixtures containing equimolar amounts of either [¹⁴C]PtdCho and unlabeled PtdIns, or [¹⁴C]PtdIns and unlabeled PtdCho, respectively, the same preference for PtdIns over PtdCho was found with single phospholipid substrates, strongly suggesting a high substrate preference of these enzymes for negatively charged phospholipids.

### DISCUSSION

Characterization of Two Novel Phospholipases B/Lysophospholipases—Sequence homology, results from overexpression, and activity analyses in vivo and in vitro strongly suggest that PLB2 and PLB3 encode phospholipases B/lysophospholipases in yeast. The most significant differences in enzymatic properties among the three phospholipases B of S. cerevisiae are reflected in their substrate specificity. The ratio of $k_{\text{eff}}$ PtdSer to $k_{\text{eff}}$ PtdEtn for Plb1p is about 1600, whereas for Plb2p this ratio is about 90. Plb3p does not accept PtdCho and PtdEtn as substrates. Interestingly, the ratio of $k_{\text{eff}}$ PtdSer to $k_{\text{eff}}$ PtdIns is around 3 for all three enzymes.

The strict specificity of activity observed upon expression of PLB3 for PtdIns and PtdSer is unique among phospholipases B. In S. pombe, a phospholipase B has been described (29) that preferentially hydrolyses PtdIns. However, PtdEtn and PtdCho are also hydrolyzed to some extent by the S. pombe enzyme, and in contrast to Plb3p from S. cerevisiae, the S. pombe enzyme does not accumulate lyso-phospholipids during the reaction in vitro.

### TABLE III

| Substrate  | PLB1 $k_{\text{eff}} \times 10^3$ | PLB2 $k_{\text{eff}} \times 10^3$ | PLB3 $k_{\text{eff}} \times 10^3$ |
|------------|----------------------------------|----------------------------------|----------------------------------|
| PtdSer     | $118 \pm 2$                      | $18 \pm 2$                       | $0.046 \pm 6$                   |
| PtdIns     | $34 \pm 3$                       | $8 \pm 1$                        | $0.014 \pm 5$                   |
| PtdCho     | $0.2 \pm 0.3$                    | $1.5 \pm 0.3$                    | ND                              |
| PtdEtn     | $0.075 \pm 0.01$                 | $0.2 \pm 0.03$                   | ND                              |
| Lyso-PtdCho|                                 | $101 \pm 18$                     | ND                              |

*a* cww, cell wet weight.  
*b* —, not determined.  
*c* ND, not detectable.

The phospholipase B produced upon expression of PLB2 has a broader substrate specificity, similar to S. cerevisiae Plb1p. It is noteworthy that PtdIns-bisP and phosphatidic acid are hydrolyzed by Plb2p and Plb1p, but not by Plb3p. Whether or not this has any bearing on the function of the signaling phospholipids, PtdIns-bisP and phosphatidic acid, needs to be established.

Phospholipases B are characterized as enzymes that sequentially remove both fatty acyl groups from diacylglycerophospholipids and therefore have both phospholipase A and lysophospholipase activities. In the case of Plb1p and Plb2p, lysophospholipase activity greatly exceeds phospholipase A activity, resulting in complete decaylation without the accumulation of lyso-lipid intermediates. In contrast, Plb3p has lower lysophospholipase than phospholipase A activity. In this respect, Plb3p resembles mammalian cytosolic phospholipase A₂, which also has lysophospholipase activity (32, 33).

Similar to Plb1p from S. cerevisiae (1) and S. pombe (29), Plb2p and Plb3p show transacylase activity in vitro and convert lyso-phospholipids to diacylglycerophospholipids (Fig. 5C). This activity may play a physiological role in vivo in the remodeling of phospholipid molecular species, given the strikingly different fatty acid patterns of cellular phospholipids derived from similar precursor molecules (16). Experiments are currently under way to elucidate the fatty acid pattern of individual phospholipids in plb1, plb2, and plb3 mutants.

Sequence homologies between yeast phospholipases B and mammalian cytosolic phospholipase A₂ suggest that these en-
zymes may act by a common catalytic mechanism. Pickard et al. (34) identified consensus motifs around putative catalytically competent amino acid residues of human cytosolic phospholipase A₂. The authors postulated a triad comprising Arg, Ser, and Thr that is conserved in cytosolic phospholipase A₂ from a number of mammalian species and in several non-mammalian phospholipases B, including S. cerevisiae Plb1p, and that is essential for catalytic activity. Recent data from x-ray analysis of cytosolic phospholipase A₂ suggest that only Ser and Asp are directly involved in catalysis, thus forming a catalytic dyad (35). It will be of interest to fully identify the catalytic center used by yeast phospholipases B. According to Pickard et al. (34), the predicted catalytic center in Plb1p consists of Arg-109, Ser-147, and Asp-403, which is also conserved in Plb2p and Plb3p at positions Arg-115 (111), Ser-153 (149), and Asp-409 (405), respectively (Fig. 1). Thus, the three yeast phospholipases B may indeed operate with the help of this new catalytic center that implies covalent catalysis via intermediary formation of an acyl-Ser complex; this mechanism is the underlying cause of the transacylase activities of the three phospholipases B.

Function of the Novel Phospholipases in Vivo—The experimental strategy to study the function of the novel phospholipases was to construct mutants in which the three putative phospholipase B genes were disrupted, and single PLB genes were re-introduced and expressed in this genetic background. By eliminating overlapping activities, each of the individual enzymes could be studied separately.

Simultaneous inactivation of the three PLB genes resulted in total loss of phospholipase B activity in vitro. A lack of an apparent growth defect associated with loss of phospholipase B activity is puzzling, but may suggest the presence of alternative pathways, as is indicated by some residual release of GroPlns and GroPCho from a strain lacking all three phospholipases B.

One possible candidate would be Spo1p, which has considerable (26%) sequence homology to Plb1p. However, Spo1p is expressed only during sporulation (36) and may thus not be active during vegetative growth. Indeed, overexpression of Spo1p results in an increase in total cellular phospholipids without significantly changing the phospholipid pattern, indicating that these activities contribute to the homeostasis of membrane phospholipids.

Extracellular phospholipases are considered potential virulence factors in pathogenic fungi (26). Given their high homology to the C. albicans enzyme, S. cerevisiae phospholipases may be adequate and easy to handle models to study inhibitors of virulent phospholipases.

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Note Added in Proof—After submission of our manuscript we became aware of a publication (Fyrst, H., Oskouian, B., Kuyper, F. A., and Saba, J. D. (1999) Biochemistry 38, 5864–5871) describing the isolation of the PLB2 gene. On the whole their results are consistent with data reported here, but some significant differences can be noted. For example, Fyrst et al. claimed that Plb2p has no transacylase activity, whereas we found significant transacylase activity which, however, strongly depended on assay conditions: transacylase activity would be consistent with the assumption that Plb2p is a serine hydrolase. Furthermore, we did not observe a significant change in growth phenotype of cells overexpressing Plb2p as reported by Fyrst et al. Presumably, differences in the genetic background result in different responses to Plb2p expression.

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