Several enzymes with lysophospholipase/phospholipase B activity have been described from the budding yeast \textit{Saccharomyces cerevisiae}. \textit{In vitro}, these enzymes are capable of hydrolyzing all phospholipids that can be extracted from yeast cells. Two forms of the enzyme have been isolated from plasma membranes and a third from culture supernatants and the periplasmic space, but their biological roles have not been determined. These highly glycosylated enzymes were reported to have very similar catalytic properties but differed with respect to apparent molecular weight. We isolated a gene from \textit{S. cerevisiae}, encoding a protein predicted to share 45% amino acid sequence identity with phospholipase B from \textit{Penicillium notatum}. This yeast gene, designated \textit{PLBl}, was mapped to the left arm of chromosome VIII.

No residual lysophospholipase/phospholipase B activity was detected upon assay of extracts or culture supernatants of a \textit{plblA} mutant. Thus, either the \textit{PLBl} gene encodes all of the previously detected isoforms of phospholipase B or its gene product is required for their expression or activation. Deletion of \textit{PLBl} did not result in any apparent phenotypic defect, suggesting either that we failed to identify the growth conditions that would betray such a defect or that \textit{Plblp} is functionally redundant with another protein, whose activity has gone undetected. A \textit{plblA} mutant released wild-type levels of the soluble phosphatidylinositol metabolite glycerophosphoinositol into the growth medium but released greatly reduced levels of the corresponding phosphatidylcholine and phosphatidylethanolamine metabolites. These results indicate that \textit{PLBl} is principally responsible for the production of the deacylation products of phosphatidylcholine and phosphatidylethanolamine but not phosphatidylinositol.

Phospholipase B has been described in a variety of eukaryotic species from fungi to mammals (1-4). Although no biological function has been defined experimentally for these enzymes, they are suspected to play a role in phospholipid turnover. Phospholipase B isoforms display several catalytic activities. (i) They release fatty acids from both the \textit{sn}-1 and \textit{sn}-2 positions of phospholipids without accumulation of lysophospholipid intermediates; (ii) they catalyze the release of fatty acids from lysophospholipids; and (iii) phospholipase B isoforms from the budding yeast, \textit{Saccharomyces cerevisiae} (and perhaps other species) possess an acyltransferase activity, catalyzing the synthesis of phospholipids from lysophospholipids (5, 6). Three forms of this enzyme have been reported in the budding yeast, \textit{S. cerevisiae}: two isolated from plasma membranes (5) and one secreted through the periplasmic space into culture supernatants (6). We report here the isolation and molecular genetic characterization of a \textit{S. cerevisiae} gene, \textit{PLBl}, encoding a protein that shares a high degree of structural similarity with phospholipase B from \textit{Penicillium notatum}. A mutant strain carrying a deletion allele of this gene is viable but has no detectable phospholipase B activity. This mutant strain produces reduced levels of choline- and ethanolamine-containing, phospholipase B-catalyzed phospholipid turnover products \textit{in vivo}. However, this strain produces wild-type levels of inositol-containing deacylation products. Thus, distinct enzymatic activities may be responsible for the deacylation of different membrane phospholipids in yeast.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, Transformations, and Nucleic Acid Manipulations**—All yeast strains used in this study (Table I) were derived from \textit{EG123 (MATa leu2-3, 112 ure2-52 his3-1 ade2-1 his4 can1") (7). Yeast cultures were grown in YEP (1 % yeast extract, 2 % Bacto Peptone) supplemented with 2 % glucose. Synthetic minimal medium (SD; Ref. 8) supplemented with the appropriate nutrients was employed to select for plasmid maintenance and gene replacements. Yeast transformation was by the lithium acetate method (9). General genetic manipulation of yeast cells was carried out as described (8). Genomic yeast DNA and plasmids were isolated and prepared for restriction endonuclease digestion and hybridization as described previously (10). Nick-translation, hybridization, and DNA sequence analysis were also carried out as described (11).

**Bacterial Strains**—DH5a (12), HB101 (13), and TG1 (14) were used for the propagation of all plasmids and plasmie. Plasmie M13mp18 and M13mp19 (15) were used to generate single-stranded template DNA for the propagation of all plasmids and phage. Plasmid M13mp18 and M13mp19 (15) were used to generate single-stranded template DNA for sequence determination. Escherichia coli cells were cultured in Luria broth or YT medium and transformed or were infected with M13 by standard methods (16).

**PLBl Gene Replacement**—A deletion mutant allele of \textit{PLBl} was constructed by the method of Rothstein (17). A 3.3-kb \textit{SpI} fragment bearing the entire \textit{PLBl} gene was first cloned into \textit{pUC}18. After digestion of this plasmid with \textit{HpaI} and \textit{BglII} to remove nearly the entire \textit{PLBl} coding sequence (2.0 kb), the ends were made flush with Klenow

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textregistered/EMBL Data Bank with accession number(s) L23089.

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1 The abbreviations used are: kb, kilobase pair(s); lysPC, lysophosphatidylcholine; DPPC, dipalmitylophosphatidylcholine; GPl, glyco- sterylphosphatidylinositol; GroPIns, glycerophosphoinositol; GroPCho, glycerophosphocholine; GroPtn, glycerophosphoethanolamine.
fragment and dephosphorylated with calf intestinal alkaline phosphatase. A 1.3-kb Smal fragment bearing the S. cerevisiae URA3 gene (from pUC18URA3; Ref. 19) was ligated into the blunt-end site of this construct. The resulting 5.2-kb SphI fragment (bearing \( pib \Delta > URA3 \)) was isolated and used to transform a diploid strain (1788) by selecting for ura3 prototrophy. Restriction and hybridization analysis of genomic DNA from the resulting transformants confirmed that translocations had occurred at the PLBl locus.

**Preparation of Phospholipase B from Culture Supernatants and Periplasmic Space**—For preparation of culture supernatants, cells from saturated YEP-glucose cultures (\( A_600 = 6.0-6.3 \)) were removed by centrifugation at 3,000 \( x \)g for 5 min. Filter sterilized culture supernatants were assayed directly or after concentration by Centricon-30 (Amicon). Phospholipase activity from culture supernatants was stable at 4 \( ^\circ \)C for several months.

Soluble phospholipase B secreted into the periplasmic space was isolated from mid-exponential phase cells (\( A_600 = 5.0 \)), grown on inositol-free medium (18). After removal of the cell wall by treatment with zymolyase, as described by Daum et al. (19), spheroplasts were removed by centrifugation (2,000 \( x \)g for 5 min). A second high speed centrifugation (50,000 \( x \)g for 1 h) removed remaining particulate material. Enzyme from these preparations was stored in liquid nitrogen.

**Preparation of Phospholipase B from Crude Membranes and Purified Plasma Membranes**—Phospholipase B was isolated from crude membranes by glass bead lysis followed by sucrose gradient centrifugation. Membranes were harvested by centrifugation (3,000 \( x \)g for 5 min), resuspended in 10% initial volume of SED (1 M sorbitol, 25 mM EDTA, 50 mM sodium citrate, pH 6.0-6.3) were removed by centrifugation (3,000 \( x \)g for 5 min) and finally resuspended in 1% glutathione (5.8). Spherical growth of microorganisms was achieved by the addition of substrate and carried out for 7.5 min at 30 \( ^\circ \)C. Reactions were terminated by addition of 400 \( \mu \)l of ice-cold chloroform/methanol (2:1, \( v/v \)) and incubated at 60 \( ^\circ \)C for 10 min. Reactions were initiated by addition of 100 \( \mu \)l of 100 mM glycine, pH 4.5, 0.01% SDS. Freshly emulsified substrate (20 \( \mu \)l) was added to plasma membrane (40 \( \mu \)g of protein) or 50 \( \mu \)l spheroplast supernatant (equivalent to periplasmic space from 7 mg of wet cells) in glycine buffer (total reaction volume of 100 \( \mu \)l). Reactions were initiated by the addition of substrate and carried out for 7.5 min at 30 \( ^\circ \)C. Reactions were terminated by addition of 400 \( \mu \)l of ice-cold chloroform/methanol (2:1, \( v/v \)). The mixture was vortexed, and phases were separated by centrifugation (5,000 \( x \)g for 5 min). Radioactivity in the aqueous phase was measured by liquid scintillation counting.

**Analysis of Extracellular Phospholipid Metabolites**—Yeast cells were grown to saturation in synthetic complete medium (22) containing 50 \( \mu \)M \([\text{3P}]\text{Pi} \), \( 75 \mu \)M inositol. Cells were removed by centrifugation, and culture supernatants were filtered prior to spotting onto white ribbon paper (no. 589, Schleicher & Schuell) for chromatography. The decacylated derivatives of yeast phospholipids were separated in two dimensions, as described by Angus and Lester (system III; Ref. 23). Migration of relevant phospholipid metabolites was verified using either radiolabeled standards (inositol, GroPIns, choline, methionine, phosphoric acid) or cold standards (GroPIns, GroPCho, GroPEtN, inositol phosphate, choline phosphate, ethanolamine phosphate) with chemical detection methods for phosphate groups (24).

**RESULTS**

**Characterization of the PLB1 Gene**—In the course of characterizing dosage-dependent suppressors of a deletion mutation of the MPK1 gene, which encodes a homologous MAP kinase (MAP kinase kit; Ref. 15), we determined the DNA sequence of a gene that is adjacent to one of the cloned suppressor genes. Suppressor gene 4 was isolated as described previously (26), in the multicopy shuttle vector YEp24 (contains S. cerevisiae URA3; provided by I. Herskowitz, University of California). The characterization of this gene will appear elsewhere. Carried on the same plasmid as suppressor gene 4 was another gene with an open reading frame encoding a polypeptide of 664 amino acids (calculated molecular mass of 73 kDa) (Fig. 1). This value assumes the use of the 5'-methionine codon in the open reading frame. A sequence identical to the consensus transcriptional control sequence TATAAA is located at position −200; however, no sequences similar to the 10-base pair consensus transcriptional control site found in many lipid metabolism genes (27) were found. No consensus sequences for intron splicing (28) were found in the sequence 5' to 300 base pairs of the predicted translational initiation site. A consensus tripartite control sequence for transcription termination (29) starts 169 base pairs 3' of the translation termination site; no common 3'-untranslated sequences were found. This predicted protein was compared with sequences in the GenBank and National Biomedical Research Foundation data bases (3) and found to possess sequence similarity to a single entry: phospholipase B from P. notatum (31). This Penicillium protein is similar in size (603 amino acids) to the predicted yeast protein. The two proteins share 45% sequence identity overall, their C-terminal halves being more closely related than...
The deletion allele (plblA::URA3) was transplaced into a diploid strain (1788) with multiple auxotrophic markers by selecting the plblA::URA3 mutant by haploid segregants was confirmed by restriction and hybridization analysis (Fig. 3). The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The PLBl protein possesses 20 potential N-glycosylation sites at positions 388, 459, 489, 513, 541, 565, 582, and 641 (33). Additionally, the predicted phospholipase requires yeast PLBI gene. The nucleotide sequence of PLBI was determined for both strands. The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The overline indicates the putative transcriptional control sequence, TATAAA.

Deletion of PLBI—To examine the phenotypic defect associated with loss of PLBI function, a deletion mutant of PLBI was constructed in vitro. A 2.1-kb fragment of PLBI, which includes nearly the entire coding sequence, was replaced with the S. cerevisiae URA3 gene (see “Experimental Procedures”). The deletion allele (plblA::URA3) was transplaced into a diploid strain (1788) with multiple auxotrophic markers by selecting for uracil prototrophy. Two independently derived Ura+ transposed alleles were isolated and sequenced. All four tetrads were dissected. All four tetrads were dissected. All four tetrads were dissected.

The putative transcriptional control sequence, TATAAA, was confirmed by restriction and hybridization analysis (Fig. 3). The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The overline indicates the putative transcriptional control sequence, TATAAA.

**Fig. 1. Nucleotide sequence and predicted amino acid sequence of the PLBI gene.** The nucleotide sequence of PLBI was determined for both strands. The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The overline indicates the putative transcriptional control sequence, TATAAA.

**Fig. 2. Alignment of the predicted PLBI-encoded protein with the P. notatum phospholipase B (P-PLB).** Identical residues are boxed. Gloves were introduced by dashes. The deduced amino acid sequence for the Penicillium protein has been published (24).
Phospholipase B Activities in Yeast—Three enzymes with similar catalytic properties to the Penicillium phospholipase B (3) have been purified and characterized previously from *S. cerevisiae* (4–6). Two forms of this highly glycosylated enzyme were isolated from plasma membranes, and a third soluble form was isolated from culture supernatants and the periplasmic space. Although these proteins differed with respect to their apparent molecular masses and carbohydrate contents, they all displayed very similar catalytic properties to one another. Moreover, the plasma membrane forms migrated with identical apparent molecular mass (67 kDa) when reduced to their constituent protein components by treatment with endoglycosidase H (5). These observations led to the suggestion that all three forms of the enzyme are derived from a common protein component by differential glycosylation (6). Each of these yeast enzymes was found to exhibit three activities: 1,2-acylhydrolase activity on diacylphospholipids (phospholipase B activity), acylhydrolase activity on monoacylphospholipids (lysophospholipase activity), and acyltransferase activity on monoacylphospholipids to form the corresponding diacylphospholipids (5, 6).

We examined the relationship between the *PLB1* gene and the previously characterized enzymes. The similarity in predicted molecular mass of the *PLB1* gene product (73 kDa) to the apparent molecular mass of the protein moiety from the purified glycoproteins suggested that *PLB1* could encode a protein responsible for one or all of the different isoforms. First, we measured the lysophospholipase activity in culture supernatants of wild-type cells (1788), wild-type cells bearing the multicopy plasmid YEpl4 (1788), which contains a functional *PLB1* copy plasmid (DL908), and *plb1Δ:URA3* cells (DL882), grown to stationary phase. Maintenance of *PLB1* in multiple copies resulted in an increase in lysophospholipase activity of approximately 17-fold over wild-type using 1-[14C]palmitoyl-glycerophosphocholine (lysoPC) as substrate (Table II and Fig. 4). The *plb1Δ:URA3* strain failed to secrete detectable lysophospholipase activity (<1% of wild-type levels). Deletion analysis of YEpl4 (*PLB1*) confirmed that the region bearing the *PLB1* gene (from the *Sppl* site to the right end of the insert; see Fig. 3) was necessary and sufficient for overexpression of this activity (data not shown).

The formation of DPPC from lysoPC (acyltransferase activity) was a relatively minor activity catalyzed by culture supernatants, as compared with the lysophospholipase activity. Although most of the 14C-labeled product comigrated with palmitic acid, approximately 10% of the product comigrated with DPPC (Fig. 4), consistent with a previous report (6). This acyltransferase activity was elevated over wild-type in culture supernatants from DL908 and was not detected in those from DL882. Because DPPC is also a substrate for these enzymes, its rate of production from lysoPC could not be measured accurately.

The purified forms of the yeast lysophospholipase were reported to display phospholipase B activity at 1–3% of the level of lysophospholipase activity (4–6). Using labeled DPPC as substrate, we detected phospholipase B activity in concentrated (20-fold) culture supernatants from DL908 at a level approximately 1% of that of the lysophospholipase activity associated with these supernatants (Table II). The level of phospholipase B activity in culture supernatants from wild-type (1788) cells and *plb1Δ:URA3* (DL882) cells was below the level of detection.

Next we examined the lysophospholipase and phospholipase B activities in crude membrane extracts. Spheroplasts were generated to eliminate any secreted phospholipase that might be trapped between the plasma membrane and the cell wall (6). Spheroplasts were lysed and washed, and phospholipase was extracted from the membranes (see "Experimental Procedures"). Membrane extracts from DL908 cells possessed approximately 6-fold higher levels of lysophospholipase and phospholipase B activities than membranes from wild-type cells (Table II). Membranes from DL882 cells possessed no detectable lysophospholipase (<0.1% of wild-type levels) or phospholipase B (<10% of wild-type levels) activities. Although we anticipated that membrane extracts might be contaminated with phospholipase A activities, which would interfere with determinations of phospholipase B activity, this was not the case. Apparently, such activities either did not fractionate with phospholipase B activity, or were inactivated during extraction. This was also true for phospholipase B activity isolated from...
purified plasma membranes (see below).

Finally, we examined the phospholipase B activity either released from the periplasmic space or extracted from purified plasma membranes. Table III shows that plasma membranes and periplasm from DL908 possessed 4- and 10-fold higher levels of phospholipase B activity than those from wild-type cells, respectively. The same fractions from DL882 were devoid of phospholipase B activity (<1% and 0.1% of wild-type levels, respectively).

Taken in the aggregate, these results indicate that the PLBl gene is responsible for the activity of all of the previously isolated forms of yeast lysophospholipase/phospholipase B. The original report describing the isolation of two forms of this enzyme from plasma membranes indicated that roughly equivalent levels of activity were derived from each form (5). Therefore, if PLBl encoded only one of the plasma membrane forms, a plb1Δ mutant should have retained detectable levels of the other activity. This was not the case, suggesting that PLBl is required for the activity of all three forms.

Analysis of Extracellular Phospholipid Metabolites—Phospholipid turnover in yeast results in the release of soluble phospholipid metabolites, such as GroPIns, GroPCho, and GroPEtn into the growth medium (23, 36). These molecules were anticipated to be among products of Plblp activity on various membrane phospholipids. Therefore we examined the release of these metabolites by a plb1Δ mutant. Wild-type (1788) and plb1Δ mutant (DL882) cells were cultivated in medium containing [32P]H3P04. Two-dimensional chromatographic analysis of culture supernatants revealed that DL882 released normal amounts of GroPIns (96% of wild-type levels) into the medium. In contrast, GroPEtn and GroPCho levels were reduced to 14% and 5% (mean of two experiments) of wild-type levels, respectively. These results indicate that although PLBl is principally responsible for the deacylation of phosphatidylincholine and phosphatidylethanolamine in vitro, another enzyme must catalyze the deacylation of phosphatidylinositol to produce extracellular GroPIns.

Mapping the Chromosomal Location of PLBl—To determine the chromosomal map position of PLBl, a probe derived from the coding sequence of this gene was hybridized to a set of λ clone and cosmids grid filters (provided by M. Olson, Washington University). The PLBl probe hybridized with clones 8270, 3731, and 6114, which correspond to an overlapping region of chromosome XIII, approximately 100-kb centromere proximal to SUP5. This also places PLBl within 40 kb of PPZ1 (26). We confirmed this tight linkage with a genetic cross between a plb1Δ::URA3 mutant and appZ1::TRP1 mutant, which yielded 18 parental ditype tetrads and 1 tetratype tetrad from 19 examined, corresponding to a genetic linkage of 2.6 centimorgans.

DISCUSSION

The PLBl Gene Is Required for the Expression or Activity of All Known Forms of Yeast Lysophospholipase/Phospholipase B—Three forms of lysophospholipase/phospholipase B have been described for S. cerevisiae. Two isoforms reside in the plasma membrane and a third soluble form is secreted through the periplasmic space into the growth medium (4–6). In this study, we have characterized a novel yeast gene, PLBl, which encodes a homolog of the P. notatum phospholipase B. The predicted yeast protein shares 45% amino acid sequence identity with the Penicillium enzyme (31). A deletion mutant of the yeast PLBl gene is devoid of detectable lysophospholipase and phospholipase B activities in the plasma membrane, the periplasm, and the culture supernatant. Thus, PLBl may encode a core protein moiety that is common to all of the detected isoforms of phospholipase B in yeast. Alternatively, the PLBl gene product is required for the expression or activation of all of these isoforms. However, since the predicted PLBl-encoded protein (Plblp) is closely related to a known phospholipase B from another organism, it appears highly probable that it is indeed the structural gene for a core protein moiety of the various isoforms.

All three known forms of yeast phospholipase B are highly glycosylated. The two plasma membrane forms have apparent molecular masses of 145 and 220 kDa (5), and the secreted form has been reported to migrate in a broad band from 200–280 kDa (6). On the basis of the similarity in catalytic properties displayed by these isoforms, a correlation between their apparent molecular masses and carbohydrate contents, and the reduction of these forms to constituent protein moieties of identical apparent molecular mass upon treatment with endoglycosidase H, it has been proposed that these isoforms arise from differential glycosylation of a common polypeptide (5, 6). The plasma membrane forms of phospholipase B may be mature products of a glycosylation pathway that differs in some way from the pathway that produces the secreted form. However, the progression in size from the plasma membrane forms
to the secreted form suggests that the former may represent intermediates in a glycosylation pathway that culminates in production of the latter.

If all three forms of phospholipase B are processed through the same pathway, the soluble form may be released from the membrane. One possibility is that phospholipase B is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Plb1p possesses a hydrophobic C-terminal signal required for GPI anchor attachment (34, 37). Moreover, Plb1p shows similarity to the S. cerevisiae Gas1 protein in the region to which a GPI anchor is attached to the latter. The modified Asn residue in Gas1p resides within a sequence (504)KKNAG(508) that is, in both cases, immediately N-terminal to the hydrophobic region. Phospholipase activity directed against a GPI anchor would release the protein from the plasma membrane and could account for the secreted form of the enzyme.

Analysis of Extracellular Phospholipid Metabolites—Yeast cells normally release the soluble products of phospholipid turnover into the medium during growth. We found that the levels of GroPCho and GroPEtn released were greatly reduced (but not eliminated) in a plb1Δ mutant relative to wild-type. In contrast, wild-type levels of GroPIns were released by this mutant. These results indicate that although the known phospholipase B isozymes can deacylate all phospholipids isolated from yeast cells in vitro (4), these enzymes are not responsible for deacylation of phosphatidylinositol and the consequent production of extracellular GroPIns in vivo.

Deletion of the PLB1 gene did not result in an apparent phenotypic defect. We may have failed to identify a specific growth condition that would have revealed a phenotypic defect associated with loss of PLB1 function. Alternatively, there may exist another form of phospholipase B that is functionally overlapping with Plb1p. Indeed, we have preliminary evidence for the existence of a lysophospholipase in yeast plasma membranes that has a pH optimum much higher than that of the previously described isozymes.9 If such an enzyme had a preference for phosphatidylinositol in vivo, but could hydrolyze other phospholipids with lower efficiency, this would also explain the observed pattern of released metabolites in the plb1Δ mutant.

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3 S. Wagner and F. Paltauf, unpublished observation.

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