Phospholipase D activity has been identified in a wide variety of cell types and organisms from bacteria (1), yeast (2), plants (3), and mammals (reviewed in Refs. 4 and 5). PLD acts to catalyze the hydrolysis of certain phospholipids, i.e. catalyzing the hydrolysis of phosphatidylcholine (PC) to yield phosphatic acid (PA) and choline. PA, itself, is thought to act as an intracellular second messenger in PLD-induced signaling processes (reviewed in Ref. 5) and may also be involved in regulating the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (6).

Activation of PLD in mammals occurs via known receptor-mediated mechanisms (reviewed in Ref. 5), and evidence exists for the involvement of protein kinase C (7), heterotrimeric GTP-binding (G) proteins (reviewed in Refs. 5 and 8), and small G proteins of the ARF and Rho families in its activation (9–12). Two distinct activation requirements for mammalian PLD have been described: one type of enzymatic activity is GTP- and ARF-dependent, while another is GTP-indepedent (13). However, both types of enzymatic activities can be stimulated by PIP$_2$ (13, 14). Interestingly, recent studies have shown that PIP$_2$ biosynthesis, itself, is a direct prerequisite for PLD activation (15), which suggests that PIP$_2$ is a co-factor of PLD.

Recent work has implicated the activation of both phosphoinositide (PI) metabolism and ARF as necessary steps for the intracellular trafficking of membrane-bound compartments (reviewed in Refs. 16–18). Since PLD activity in mammals may be activated by either ARF or PIP$_2$, it is intriguing to think that PLD could play an important role in mediating membrane trafficking. Yet, despite the wealth of information concerning the biochemical properties of PLD, little is known about the structural and molecular properties of the enzyme(s), while even less is known about its biological role. To date, the only published sequence for a protein from eukaryotes having PC-specific PLD activity is from plants (3), and its role in either cell signaling or membrane trafficking remains virtually unknown.

Here we describe the identification and characterization of a gene encoding a PLD from yeast. We show that the yeast enzyme is homologous to plant PLD and shares some homology with a bacterial phosphatidylinositol transferase. Disruption of the PLD1 gene in yeast has no effects upon cell growth and morphology during vegetative growth but is likely to be required for meiosis and sporulation.

EXPERIMENTAL PROCEDURES

Chemicals—PIP$_2$ was obtained from Sigma. 1-Palmitoyl-2-[6-(7-nitrobenzo-2-1,3-diazol-4-yl)amino]caproyl-phosphatidylcholine (C$_6$-NBD-PC) was purchased from Avanti Polar Lipids.

DNA Manipulations—DNA restriction endonucleases, Taq polymerase, and T4 DNA ligase were used as recommended by the suppliers (Boehringer Mannheim, MBI Fermentas). Molecular cloning techniques were performed as described by Sambrook et al. (19).

Media and Genetic Manipulations—Yeast were maintained on standard growth media containing 2% glucose as a carbon source. Amino acid-rich medium (YPD (yeast extract and Bactopeptone containing 2% glucose)) and YP (yeast extract and Bactopeptone), synthetic minimal medium (SC), and SC drop-out medium, lacking an essential amino acid or nucleotide base, were used. Drop-out medium was used to maintain plasmid selection. Media were prepared according to Rose et al. (20). SC complete and drop-out media consisted of 0.7% yeast nitrogen base supplemented with the appropriate auxotrophic requirements. Other growth media included: YPA (YP containing 0.05% glucose and 2% potassium acetate); YPEG (YP containing 2% ethanol and 2% glycerol); and YPG (YP containing 3.5% galactose). Standard methods were used for the introduction of DNA into the various yeast strains, the preparation of genomic DNA, and for tetrad dissection (20). Escherichia coli strain XL-1 blue was used for plasmid transformations and plasmid DNA preparations.

Yeast Strains—Disruptions of PLD1 were made in wild-type strains J C1 (MATa ade8 can1 his3 leu2 lys2 trpl ura3) (21) and C2 (MATa ade8 can1 his3 leu2 lys2 trpl ura3) (22). Plasmid strains created included: PLD1 (MATa ade8 can1 leu2 lys2 trpl ura3) (23); PLD2 (MATa ade8 can1 leu2 lys2 trpl ura3) (24); PLD2FL1 (MATa ade8 can1 leu2 lys2 trpl ura3) (25); PLD2FL2 (MATa ade8 can1 leu2 lys2 trpl ura3) (26); and PLD2FL5 (MATa ade8 can1 leu2 lys2 trpl ura3) (27).

*This work was supported by grants from the Israel Science Foundation (to M. L.) and the Forchheimer Center for Molecular Genetics (to J. E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: PC, phosphatidylcholine; ARF, ADP-ribosylation factor; C$_6$-NBD-PC, 1-palmitoyl-2-[6-(7-nitrobenzo-2-o,1,3-diazol-4-yl)amino]caproyl-phosphatidylcholine; ORF, open reading frame; PA, phosphatic acid; PC, phosphatidylcholine; PI, phosphoinositide; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; kb, kilobase(s); bp, base pair(s).
Identification and Characterization of Yeast PLD1 Gene

Plasmids—Cosmid pEKGO13, which contains a genomic fragment from chromosome XI in vector pWE15, was a gift from A. Urrestarazu (Universite Libre de Bruxelles). This cosmid contains ~36 kb, which maps to the small arm of chromosome XI and encompasses mapped genes spo4 and tif1. Disruption constructs for PLD1 were created by subcloning a 5.1-kb EcoRI/Xhol fragment containing the coding region of PLD1 beginning at bp 837 and ending at bp -912 downstream of the termination codon into the EcoRI/Sall sites of pUC118 to yield pPLD37–5094. This plasmid was then digested with the Sall endonuclease to release an 1153-bp fragment (bp 3237–4390), which is thought to encode part of the catalytic domain of Pld1 (see Fig. 1, homology boxes 6 and 7) and was blunt-ended using standard techniques. A blunt-ended BamHI-out 1.7-kb fragment, including the HIS3 auxotrophic marker was then subcloned into yeast plasmid pPLD1::HIS3, then ligated into yeast with Klenow fragment of DNA polymerase, and religated to yield a frameshift disruption plasmid, pPPLDFS::HIS3. All plasmids were verified by restriction mapping.

To create disruptions of PLD1 in yeast, plasmids pΔPLD1::HIS3 and pΔPPLDFS::HIS3 were digested with EcoRI and Spfl to release fragments containing the disrupted PLD1 gene. These were transformed into yeast cells using standard procedures to create the strains described above. All disruptions were verified by Southern analysis.

Phospholipase D Assay—Total cell lysates from yeast grown at log phase were prepared as described previously (22), except that the lysate buffer contained 35 mM HEPES pH 7.0, with no added detergent. Lysates were cleared by low speed centrifugation (16,000 g for 0.25 min) to remove nuclei and cell wall debris. Cell lysates were flash frozen and stored at −70 °C.

The hydrolysis of C6-NBD-PC in lysates was monitored by the production of PA, essentially as described by Danin et al. (23). Briefly, the reaction mixture contained 0.3 mg/ml protein, 35 mM HEPES pH 7.2, 150 mM NaCl, 0.4 mM C6-NBD-PC, and 4 mol % PIP2. Reaction volume was 120 μl, and the reactions were incubated at 30 °C for 30 min. Transphosphatidylation assays contained between 0 and 500 μM primary alcohol as substrate. The assays were terminated by chloroform/methanol/HCl extraction (100:100:0.6), and the phospholipid products were separated by thin layer chromatography (TLC), as described (23). Fluorescent phospholipids were visualized by UV illumination, scraped from TLC plates, methanol-extracted, and counted in a fluorimeter (excitation at 468 nm; emission at 520 nm). Results are expressed as the average of two duplicate samples measured in arbitrary fluorescence units.

RESULTS
Identification of a Yeast Homolog of Plant PLD—Recent identification of a PC-specific PLD from plants allowed us to screen the National Center for Biotechnology Information (NCBI) database for sequences having direct homology with plant PLD. A previous study has shown (24) that the yeast genome sequencing project (ORF YKR031c) contains a genomic fragment from chromosome XI by the GCN3-DAL80 intergenic region and near the previously mapped spo4 and tif1 genes.

Sequence comparison between the proteins showed that they had low overall homology. However, at least eight specific regions of 11–42 amino acids in length were found to be homologous, ranging from 50 to 76% (Fig. 1). The percentage of shared residues in these regions varied between 20 and 62% (Fig. 1). One region, in particular, was also identified as having homology to a phosphatidylserine synthase from bacteria (Fig. 1, homology box 7). Thus, ORF YKR031c seemed to be a highly likely candidate for a yeast protein having PLD activity and was tentatively termed Pld1.

Identification of ORF YKR031c as a Phospholipase D—In order to verify that this ORF encodes a yeast PLD homolog, we constructed haploid and diploid yeast strains lacking PLD1 (see "Experimental Procedures"). Yeast-bearing disruptions in PLD1 were found to be morphologically normal and grew at rates similar to wild-type cells grown under vegetative conditions (data not shown). However, these pld1Δ cells were found to be temperature-resistant like wild-type cells (data not shown).

In order to verify that pld1Δ cells lack PLD activity, we performed PLD assays in vitro on yeast cell lysates, using C6-NBD-PC as a substrate. Wild-type cells of either mating type were found to have significant levels of PLD activity, as judged by PA production (Fig. 2). In contrast, pld1Δ cells were found to have no detectable PLD activity, indicating that the disruption of PLD1 destroys the protein responsible for PLD function. Thus, the PLD1 gene product (Pld1) is likely to confer PLD-like activity.

Characterization of Pld1 Activity—A previous study has characterized biochemically PLD activity in yeast (2), which activity localizes primarily to membrane fractions and was found to be both PC-specific and capable of utilizing short chain alcohols in the transphosphatidylation reaction (2). Moreover, the rank order of straight and branched chain alcohols in the transphosphatidylation reaction was similar to that already described for mammalian cell PLDs. Finally, it was shown that maximal PLD activity in yeast could be induced in a cycloheximide-independent manner by shifting cells to sporulation-inducing medium, which contains acetate (2).

To verify that the activity of the PLD1 gene product is similar to that described previously, we have characterized PLD activity in wild-type and Δpld1Δ cells (Fig. 3). We first examined the effect of primary alcohol addition upon PA production and alcohol transphosphatidylation in haploid cells. We found that an increase in alcohol chain length resulted in a decrease in the

Fig. 1. Homologous regions of ORF YKR031c and plant PLD.

The diagram in A illustrates ORF YKR031c. Numbers above the diagram indicate those regions (boxed boxes) ranging between 11 and 42 amino acids that are highly homologous to regions of plant PLD. Beneath each box is the percentage identity shared between residues of YKR031c and plant PLD within that designated region. The corresponding number of amino acids for each given region is: 1–25, 2–20, 3–19, 4–42, 5–28, 6–11, 7–37, and 8–24, respectively. B shows sequence comparisons between plant PLD and YKR031c within boxes 4, 5, and 7. An additional comparison between YKR031c and phosphatidylserine synthase from E. coli (PSS) is shown. Numbers correspond to amino acid sequence of the proteins; identities are marked with a vertical bar, and conserved residues are marked with a colon.

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overall activity of the enzyme, which was characterized by both a decrease in PA formation and an increase in phosphatidyl alcohol production (Fig. 3A). Next, we examined the effect of various agents and carbon sources upon PLD activity. The addition of chelators of divalent metal cations or oleate resulted in a significant decrease in PLD activity (Fig. 3A). Likewise, the omission of PIP2 led to a substantial decrease (>65%) in activity (Fig. 3A). Thus, the PLD activity examined here is highly similar to that described previously (2) and is similar to the oleate-independent PLD from mammals (13).

In contrast, growing cells in the presence of non-fermentable carbon sources, such as acetate and ethanol/glycerol, or in the presence of galactose resulted in an increase in PLD activity over cells maintained continually in glucose. Thus, the yeast enzyme may be under partial glucose-repressible control (Fig. 3B).

Since yeast PLD is stimulated in the presence of acetate-containing medium (this study and Ref. 2), we examined whether PLD activity is increased in homologous Δpd1::HIS3/Δpd1::HIS3 and heterozygous Δpd1::HIS3/PLD1 cells shifted from glucose-containing medium to acetate-containing medium (sporulation conditions). Cells bearing homologous disruptions in PLD1 showed little basal activity when grown in glucose-containing medium or when shifted to acetate-containing medium. In contrast, heterozygous Δpd1::HIS3/PLD1 diploid cells showed a strong time-dependent induction of PLD activity that peaked within 2 h of shifting the cells to acetate-containing medium (Fig. 3B and data not shown). Thus, PLD activity is induced under sporulation conditions in a PLD1-dependent fashion.

The stimulation of PLD activity upon transfer to acetate-containing medium suggested to us that a functional PLD might be required for sporulation in yeast. Indeed, diploid yeast bearing homozygous disruptions of PLD1 (Δpd1::HIS3/Δpd1::HIS3) were found to be unable to undergo sporulation to yield ascii under conditions by which wild-type cells are known to sporulate (data not shown). Thus, there appears to be a requirement for PLD1 in the sporulation of yeast cells.

**DISCUSSION**

We have identified a yeast protein that confers PLD activity to yeast cell lysates. The gene encoding this activity, PLD1, localizes to the GCN3-DAL80 intergenic region of the short arm of chromosome XI and is the region that the spo14 sporulation mutation maps to. Our results indicate that PLD1 encodes a PC-hydrolyzing PLD that confers the significant, if not total proportion, of PLD catalytic activity in yeast cells. Moreover, this activity is stimulated during sporulation conditions and is similar in requirements to that described previously by Ella et al. (2). In addition, we have demonstrated that yeast Pd1 is stimulated by PIP2, like mammalian PLD (14).

Although the true role of PLD1 is not yet known, strong evidence links PLD function to meiosis and sporulation in yeast.
cells. First, PLD1 activity is stimulated during the shift to sporulation-inducing medium (this study and Ref. 2). Second, Δpld1 cells fail to undergo sporulation. Third, while this manuscript was in preparation, the sequence of SPO14 was deposited into the NCBI database (accession number L46807) and although it was not identified as having PLD activity, we and others have now identified it as being allelic to PLD1.2 Thus, PLD activity appears necessary for yeast cells to complete the meiotic cycle.

When diploid yeast are deprived of nitrogen and fermentable carbon sources they enter a meiotic cycle that leads to the development of four haploid nuclei. These nuclei are later enveloped by a membranous spine at the spindle pole (reviewed in Ref. 25). The spine wall consists of four layers, including at least two spine-specific layers that are induced only during the meiotic cycle. SPO14 was previously identified as a sporulation-specific gene that is required for the commitment to meiotic development as well as for meiotic segregation and spore formation (26). Cells bearing the spo14 mutation are defective in sporulation but can return to mitotic division even from the late stages (i.e., tetraploid stage) of meiotic development (27). In all likelihood, then, Spo14/Pld1 may be a critical component of the final stage of the meiotic cycle, spore wall assembly.

Converging lines of evidence have implicated PLD function with PI metabolism and membrane trafficking in mammals (17). Activation of PLD by ARF, which itself acts to recruit cytosolic coat proteins to newly forming vesicles in the Golgi (reviewed in Ref. 18), may be an important mechanism for PI metabolism and membrane trafficking in mammals. Although this work is still in its infancy, yeast may prove to be an ideal system in which to assay the role of PLD upon PI metabolism, as well as in cell signaling and membrane trafficking. Interestingly, our work already demonstrates that Pld1 activity is not necessary for vegetative cell growth, which implies that membrane trafficking processes like secretion are not prominently affected. Thus, we predict that Pld1 function is not likely to be required for the anterograde trafficking of secreted essential proteins, although it could play a role in other trafficking steps.

Acknowledgments—We thank Dr. A. Urrestarazu for the gifts of cosmids and bacteria. We are also grateful to Drs. J. Engebrecht and A. Morris for sharing unpublished results.

Note Added in Proof—After this work was accepted, a paper describing the identification of a human PLD gene was published (Hammond, S. M., Altshuller, Y. M., Sung, T.-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29643). This gene is highly homologous to yeast PLD1.

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2 This study and J. Engebrecht, personal communication.