A gene encoding phospholipase D (PLD) in *Saccharomyces cerevisiae* was identified. The 195 kDa product of *PLD1* has 24%, overall sequence identity with a plant PLD. Expression of yeast PLD activity was eliminated by one-step gene disruption. Yeast haploids lacking PLD activity were deficient in growth on non-fermentable carbon sources. Diploids lacking expression of *PLD1* were unable to sporulate.

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phospholipids to produce phosphatidic acid (PA) [1,2]. The preferred substrate for the mammalian enzyme is phosphatidylcholine (PC), an abundant membrane phospholipid. A distinguishing feature of the PLD reaction is the ability of the enzyme to perform 'transphosphatidylation' in which a phosphatidylalcohol is produced in the presence of simple alcohols. In mammalian cells, PLD is activated in response to a variety of stimuli, many of which also activate phosphatidylinositol-specific phospholipase C [3–5]. Possible roles of PA include its ability to serve as a substrate for generation of diacylglycerol (DAG) by PA phosphohydrolase, or of lysophosphatidic acid by phospholipase A₂. A mitogenic role has been ascribed to PA in some studies [6–8], but not in others [9]. The sequence of a soluble PLD from the plant *Ricinus communis* (castor bean) was recently determined [10]. A mammalian form of PLD has not yet been sequenced, although a 195 kDa form has been purified to homogeneity from lung [11]. Mammalian PLD may exist in more than one form [12,13]. PLD can be regulated by G-proteins, including ADP-ribosylation factor ('ARF') [14,15], rho [16,17], and ras [18], and may also be regulated by phosphorylation [19].

In previous work we had identified PLD activity in *Saccharomyces cerevisiae* [20]. Activity was detected in yeast membranes by an *in vitro* assay utilizing a fluorescent alklyglycerophosphocholine as substrate [21]. This assay, which was originally developed for mammalian cell extracts, is capable of detecting PLD activity from plants as well as in a variety of mammalian cells and tissues, including lung [21]. The yeast PLD activity was detected in the absence of added G-proteins or guanine nucleotides. The ability of yeast PLD to perform transphosphatidylation, utilizing a series of alcohols, is similar to that of mammalian PLD. Yeast PLD is activated in response to nutritional signals [20]. Growth on non-fermentable carbon sources produces a partial activation, while growth on non-fermentable carbon sources in nitrogen-deficient medium results in a greater activation. The latter treatment, in diploid cells, results in sporulation. These results suggested that yeast expresses a form of PLD related to that present in mammalian cells, and that this enzyme plays a role in adaptive responses in yeast.

Here we identify a gene encoding PLD activity in yeast. Deletion of this gene is shown to affect the haploid-yeast phenotype with respect to nutrient utilization and diploid-yeast phenotype with respect to sporulation. We have designated this gene *PLD1*.

EXPERIMENTAL

Yeast strains

The yeast strains used in the present study were as follows: EG123, *MATa can1 his4 leu2 trp1 ura3*; SF1, *MATa his3 his4 leu2 trp1 ura3 MFα 1-lacZ*; KE1, SF1 with *pld1::LEU2*; KE2, EG123 with *pld1::LEU2*; SF199, EG123 × 246-1-1; 246-1-1, *MATa can1 his4 leu2 trp1 ura3*; 227a, *MATa cry1 lys1*; AM227a, *MATa lys1*.

Subcloning of a fragment of a *PLD1* gene from yeast

Oligonucleotide primers were synthesized at the University of Wisconsin-Madison. PCR was carried out with phage DNA extracted from A.T.C.C. clone 70452. This clone, *λMG-3*, carries an 18.6 kb fragment of chromosome XI containing the open reading frame (ORF) YKR031c identified in the yeast genome-sequencing project (accession no. Z28256). The *PLD1* gene ORF represents 5.8 kb of this sequence, or approx. 6.4 kb, including the promoter region. PCR was carried out with each primer, using the N-terminal primer 5’ TTA TAG GGT CTG CTA ATA TTA ACG AAA GAT CAC AAC TGG GTA 3’ and the C-terminal primer 5’ GAC TAG TCG AAA ATA CAG GTA.
ATG GTG T 3'. PCR reactions were carried out with 5 min of denaturation at 94 °C, 2 min at 55 °C and 2 min at 72 °C, for 25 cycles, followed by one cycle for 4 min at 72 °C. The expected PCR product of 1.8 kb was directly cloned into pCRII according to the supplier's (Invitrogen) protocol to yield plasmid pCQ1.

**Preparation of pld1::LEU2 yeast strains**

The 1.8 kb fragment was released from the pCQ1 by digestion with EcoRI and subcloned into pBluescript II SK+ vector to yield pCQ2. The 651 bp BgII fragment of the 1.8 kb insert was replaced by a 2.9 kb BglII fragment of Yep13 that contains the LEU2 gene to yield pCQ3. The PLD1 gene was disrupted by transforming S. cerevisiae strains SF1 and EG123 with pCQ3 that had been digested with Apal and Smal using lithium acetate [22]. Transformants were selected by plating on SC-Leu medium [23]. Proper integration was confirmed by Southern-blot analysis. The resulting α-haploid strain was designated KE1 and the a-haploid strain was designated KE2. A homozygous diploid was generated by mating KE1 and KE2. Zygotes were micromanipulated from mating mixtures consisting of KE1 and KE2 cells on YPD plates. The diploids were tested for the ability to mate against tester strains, production of α-factor in a halo assay, and expression of MFx 1-lacZ by growth on 5-bromo-4-chloroindol-3-yl β-d-galactoside ("Xgal") plates to confirm that the strains were diploid.

**Assay for PLD activity**

PLD activity was assessed as described previously [20]. Briefly, membranes were prepared from yeast cells following breakage of the cell wall by glass beads. PLD activity was measured in cell membranes in an *in vitro* assay utilizing BPC (BODIPY-PC; 2-decanoyl-1-O-[11(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino]undecyl)-sn-glycero-3-phosphocholine) as substrate. Each reaction mixture contained 10 µg of membrane protein; protein concentrations were determined by a Coomassie Blue binding assay (Pierce). The incubation was carried out for 60 min at 30 °C. Following separation by TLC, reaction products were imaged and quantified using a FluorImager (Molecular Dynamics).

**Assays for mating and sporulation**

The ability of the pld1::LEU2 mutants to mate was tested by a patch-mating assay [24]. Patches of cells to be tested were replica plated on to lawns of strain 227a (MATa) or AM227a (MATa) on SD minimal plates, then incubated at 30 °C for 4 days. The ability of homozygous pld::LEU2 mutants to sporulate was measured both qualitatively and quantitatively. Eight independent pld1::LEU2/::LEU2 diploids and a wild-type diploid strain were patched on to minimal sporulation medium and incubated at 30 °C for 4 days. Cells from each patch were stained with the spore-specific stain Malachite Green oxalate and counterstained with safranin O [24] to enhance the detection of rare ascis. Stained cells were examined microscopically. One of the mutant diploids and the isogenic wild-type diploid were induced to sporulate in liquid medium as described previously [20]. Mature ascis were counted by phase-contrast microscopy.

**Infection of bacteria with λMG3**

*Escherichia coli* (C-600) was grown in Luria–Bertani medium containing 10 mM MgSO4 and 0.2 % maltose to an A600 of 0.5. The bacteria were then infected with λMG3, diluted $10^{-1}$–$10^{-10}$ from a stock of 10⁷ plaque-forming units/ml, and incubated overnight at 37 °C with shaking. The cells and lysates were briefly sonicated and the 100000 g pellets were then assayed for PLD activity using the *in vitro* PLD assay described above.

**RESULTS**

**Identification of a gene encoding PLD in yeast**

A gene encoding a 95 kDa soluble form of PLD from *R. communis* was recently identified [10]. Regions of this sequence that are critical for enzymic activity have not yet been identified. In a search of available sequence databases, regions of high amino acid homology between the *Ricinus* sequence and that of a putative yeast protein were noted (24 % identity, 52 % similarity). The yeast sequence, encoding a putative ORF in chromosome XI (YKR031c), was identified by the yeast genome project. The hypothetical protein encoded by this ORF has a molecular size of 195 kDa (1683 amino acids). This size is nearly identical with that of a PLD recently purified to homogeneity from mammalian lung [10]. The *in vitro* assay for PLD activity used here detects PLD activity from yeast, plants and lung [20, 21]. Taken together, these data suggested that the yeast ORF might encode a form of PLD.

The results of predicted protein sequence homology are shown in Figure 1. The regions of highest homology are located in the C-terminal third of the yeast ORF sequence. This region contains a stretch of eight amino acids that are identical between the *Ricinus* and putative yeast sequences (residues 1108–1115 of the yeast ORF).

The fragment of yeast chromosome XI containing the entire ORF was available as a lambda clone from the collection prepared by Olson and Riles (A.T.C.C. 70452). Oligonucleotide primers were designed for PCR amplification of the desired sequence (Figure 2). Primer 1 corresponded to the sequence encoding amino acids 1108–1120 of the putative yeast ORF. Primer 2 corresponded to a region 3' to the termination codon of the hypothetical yeast protein. These primers were successfully used to amplify the expected 1.8 kb sequence (results not shown). This sequence was used to disrupt the PLD1 gene.

One-step gene replacement via homologous recombination was used to construct a haploid strain with a deletion/disruption of the putative PLD1 sequence. Successful disruption of the gene was demonstrated by Southern blotting, using the 1.8 kb gene fragment as probe (results not shown).

Expression of PLD activity was examined in membranes prepared from the parental and recombinant strains (Figure 3a). In this *in vitro* assay system, PLD activity is measured by production of both phosphatidylbutanol (PBT) and phosphatidic acid (PA) in the presence of butanol [20]. The results show that PLD activity was present in the parental strain, but absent in the recombinant a strain. Similar results were seen for a haploid and diploid strains (results not shown). PLA2 activity, as detected by production of lyso-PC, was present in both strains. Production of diacylglycerol (DAG) was eliminated in the recombinant strain, suggesting that DAG arises via the action of PA phosphohydrolase on PA in this preparation. PA phosphohydrolases have been previously described in yeast [26]. These findings confirm that the ORF encodes a gene required for expression of PLD activity in yeast.

To confirm that the gene encoded a form of PLD, bacteria were infected with λMG3 containing the complete coding sequence for PLD1. As shown in Figure 3(b), membranes from the cells infected with λMG3 contained PLD activity, as measured by phosphatidylbutanol production, while cells infected with an empty vector expressed no PLD activity. In the experiment shown, 7 % of the substrate was converted into PBT, as compared...
The deduced sequence of the putative protein encoded by the yeast PLD1 gene is aligned with the sequence determined for plant PLD. The sequence alignment was accomplished using the DNAStar program. Only the portions of sequence showing homology are presented; this includes essentially all of the plant sequence and 57% of the yeast sequence. Lines indicate identical amino acids, two dots indicate highly conservative substitutions, and one dot indicates less conservative substitutions. The sequence used to design PCR primer 1 is indicated. The sequence 1108–1120 of the yeast ORF was used to design PCR primer 1.

In (a), membranes were prepared from wild-type (WT) or pld1::LEU2 (PLD−) haploid yeast grown in complete medium. PLD activity was assessed, using an in vitro assay with BODIPY-PC as substrate. Each reaction mixture contained 10 µg of membrane protein and 1% butanol. The TLC separation of the reaction products is shown, as imaged using a FluorImager and printed with reverse contrast. The positions of diacylglycerol (B-DAG), phosphatidylbutanol (B-PBt), phosphatidic acid (B-PA), BODIPY-PC (B-PC), and lyso-glycerophosphocholine (B-lyso-PC) are indicated. The small spot running immediately above B-PC is an unidentified contaminant. In (b), lysates were prepared from E. coli incubated with (λMG3) or without (Control) λMG3. The viral stock was diluted by a factor of 10−7 for the infection. PLD activity was assessed in the membrane fraction, as described for (a). The reaction product migrating below the substrate is unidentified, but migrates similarly to B-lyso-PC [21].

with approx. 10% conversion for yeast membranes [20]. Similar results were seen with at least six separate aliquots of infected cells. Production of lyso-PC, seen in both uninfected and infected cells, was likely due to bacterial phospholipase B activity. These data indicate that PLD1 encodes a protein with PLD activity.

Phenotype of yeast lacking PLD activity

The phenotype of the pld1::LEU2 yeast strain was examined. The pld1::LEU2 mutants were able to mate successfully with tester strains, with an efficiency qualitatively similar to that of wild-type strains. To investigate further the role of PLD in yeast, a diploid pld1:LEU2 strain was generated. The ability of this strain to sporulate was assessed. When wild-type diploids were induced to sporulate by the regimen previously described [20], 25.2% of the cells sporulated and formed mature ascii within 48 h. In the same experiment, the diploids lacking PLD activity were unable to sporulate. The ORF in YKR031c

Figure 1 Sequence homology between Ricinus and yeast PLDs

The deduced sequence of the putative protein encoded by the yeast PLD1 gene is aligned with the sequence determined for plant PLD. The sequence alignment was accomplished using the DNASTar program. Only the portions of sequence showing homology are presented; this includes essentially all of the plant sequence and 57% of the yeast sequence. Lines indicate identical amino acids, two dots indicate highly conservative substitutions, and one dot indicates less conservative substitutions. The sequence used to design PCR primer 1 is indicated. The sequence 1108–1120 of the yeast ORF was used to design PCR primer 1.
form asci (sporulation frequency < 6 x 10⁻⁵). These data further support a role for PLD in sporulation in this organism.

**DISCUSSION**

In the present study we have identified a gene required for the expression of PLD activity in yeast. Cells lacking expression of this gene show a complete lack of PLD activity in our *in vitro* assay system. While it is possible that yeast expresses other forms of PLD that are not detected by this particular assay, this seems unlikely in view of the fact that a PLD with transphosphatidylidation activity has not been otherwise identified in yeast. There is a high degree of homology between the *PLD1* gene product and the catalytically active product of the *Ricinus* PLD gene. Nonetheless, the possibility that *PLD1* encodes a regulatory protein required for the expression of PLD activity was considered. The possibility appears unlikely in view of the large size of the *PLD1* gene product, its lack of homology to G-proteins or protein kinases, the fact that the targeted disruption interfered only with the C-terminal third of the molecule, and the observation that heterologous expression of the *PLD1* gene confers PLD activity to bacteria. We therefore conclude that this gene encodes an enzyme with PLD activity.

The fact that stretches of identical amino acids are present in PLDs expressed by both plants and yeast suggests that these sequences may represent a consensus sequence for PLDs. Until the regions of the enzyme involved in substrate recognition and catalysis have been identified, and PLDs have been sequenced from additional species, this conclusion remains speculative. However, it should be noted that the *Ricinus* and putative yeast PLDs differ considerably in their overall size (95 as against 195 kDa) and in other regions of their sequence. The only form of PLD purified to homogeneity from a mammalian source was shown to migrate with a molecular size of 195 kDa on SDS/PAGE. Mammalian forms of PLD appear to be subject to complex regulation involving both G-proteins and/or phosphorylation. The rapid increase in yeast PLD activity that we have observed in response to nutrients is not dependent on protein synthesis [20], suggesting that either form of regulation could occur in yeast cells. The *Ricinus* enzyme, in contrast, is a soluble protein whose regulation has not yet been established.

We have no evidence that the yeast PLD is regulated by G-proteins, and are able to detect activity of the detergent-solubilized enzyme following partial purification (K. M. Ella and K. E. Meier, unpublished data). It is not yet clear whether all PLDs can utilize diradylglycerophosphocholines possessing an alky chain linkage at the 1-position of the glycerol backbone, as is present in the substrate used in our *in vitro* studies. All PLDs studied in our laboratory (plant, yeast and mammalian) show similar utilization of alcohols as substrates for the transphosphatidylidation reaction [20]; K. M. Ella, G. P. Meier and K. E. Meier, unpublished work), suggesting that an alcohol-binding site may be conserved between species. Other sites likely to be conserved between PLDs include the phosphate- and choline-recognition sites.

In mammalian cells, PLD has been postulated to be involved in mitogenic responses [3–5]. PA produced via the PLD reaction is proposed to serve as a substrate for generation of DAG to in mitogenic responses [3–5]. PA produced via the PLD reaction is proposed to serve as a substrate for generation of DAG to

**REFERENCES**

Received 15 November 1995/18 December 1995; accepted 21 December 1995