RESEARCH COMMUNICATION

Characterization of *Saccharomyces cerevisiae* deficient in expression of phospholipase D

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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 alkylglycerophosphocholine 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, *MATα can1 his4 leu2 trp1 ura3*; SF1, *MATα 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, *MATα can1 his4 leu2 trp1 ura3*; 227a, *MATα cry1 lys1*; AM227α, *MATα 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

Abbreviations used: BODIPY (or 'B'), 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; BPC, 1-acyl-2-alkyl-1-BODIPY-glycerophosphocholine; DAG, diacylglycerol; ORF, open reading frame; PA, phosphatidic acid; PBt, phosphatidylbutanol; PC, phosphatidylcholine; PKC, protein kinase C; PLA₂, phospholipase A₂; PLD, phospholipase D; YEP, yeast extract peptone.

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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 *BglII* fragment of the 1.8 kb insert was replaced by a 2.9 kb *BglII* fragment of YEpl3 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 *ApaI* and *SmaI* 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 *a*-factor in a halo assay, and expression of *MF α 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 μ g 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 AM227 α (*MAT α*) on SD minimal plates, then incubated at 30 °C for 4 days.

The ability of homozygous *pld1::LEU2* mutants to sporulate was measured both qualitatively and quantitatively. Eight independent *pld1::LEU2/pld1::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 saffranin O [24] to enhance the detection of rare asci. 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 asci were counted by phase-contrast microscopy.

Infection of bacteria with λ MG3

Escherichia coli (C-600) was grown in Luria–Bertani medium containing 10 mM MgSO₄ and 0.2 % maltose to an *A*₆₀₀ of 0.5. The bacteria were then infected with λ MG3, diluted 10^{−1}–10^{−10} from a stock of 10⁷ plaque-forming units/ μ l, and incubated

overnight at 37° 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 regions 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 PLD. 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 α 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 λ MG3 containing the complete coding sequence for *PLD1*. As shown in Figure 3(b), membranes from the cells infected with λ MG3 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

form asci (sporulation frequency $< 6 \times 10^{-5}$). 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 transphosphatidylase 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 alkyl 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 transphosphatidylase 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 support activation of protein kinase C [27,28]. PA itself has the ability to activate protein kinases [29,30] and G-proteins [31,32] *in vitro*, although the roles of these responses in intact cells remain to be determined. Another potential product of PA metabolism is lysophosphatidic acid, which appears to bind to a G-protein-coupled receptor to induce cell spreading, mitogenesis

and inflammatory responses [33,34]. PA-utilizing forms of PLA_2 are expressed in platelets and brain [35,326]. While we have not identified this enzyme in yeast cell membranes, a PC-utilizing PLA_2 appears to be present (see Figure 3).

The exact role of PLD in nutritional sensing in yeast remains unclear. The pathways regulating carbon metabolism in this organism have not been well delineated and are not obviously analogous to pathways utilized in mammalian cells. PLD activity was previously detected in yeast mitochondria [37–39], where it was reportedly activated in response to glucose repression. However, as the transphosphatidylase activity of this form of PLD was not investigated, and its pattern of regulation is different, its relationship to the enzyme described here remains to be established.

In the region of the yeast genome containing the *PLD1* gene there are three other genes required for sporulation: *spo14*, *spo15* and *spoT23* [40]. A comparison of the restriction maps of these sporulation genes to *PLD1* revealed that *PLD1* is the same as *SPO14*. Subsequent to our designation of this gene as *PLD1* in the yeast genome database, the sequence of the *SPO14* gene was deposited in Genbank by Esposito and co-workers (accession no. L46807). Their sequence of *SPO14* matches the sequence of *PLD1* with one minor change: the sequence *SPO14* in Genbank indicates an incorrect start site for translation. The *SPO14* sequence designated the methionine at residue 304 as the initiating codon. This information is consistent with the inability of diploid *pld1::LEU2* yeast to sporulate. The observation that activation of PLD is an early response to the nutritional change leading to sporulation [20] suggests that PLD activity is required for sensing the nutritional signals that initiate sporulation.

In conclusion, the results reported here indicate that PLD activity is important in yeast cell regulation. As has been the case for other signal-transduction pathways [e.g. MAPK (mitogen-activated protein kinase)] [41], yeast may prove a useful tool for the further analysis of proteins involved in PLD-dependent signalling in mammalian cells.

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REFERENCES

- Heller, M. (1978) Adv. Lipid Res. **16**, 267–326
- Kanfer, J. N. (1980) Can. J. Biochem. **58**, 1370–1380
- Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
- Liscovitch, M. (1991) Biochem. Soc. Trans. **19**, 402–406
- Zeisel, S. H. (1993) FASEB J. **7**, 551–557
- Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. and de Laat, S. W. (1986) Nature (London) **323**, 171–173
- Fukami, K. and Takenawa, T. (1992) J. Biol. Chem. **267**, 10968–10993
- Kondo, T., Inui, H., Konishi, F. and Inagami, T. (1992) J. Biol. Chem. **267**, 23609–23616
- Jones, L. G., Ella, K. M., Bradshaw, C. D., Gause, K. C., Dey, M., Wisheart-Johnson, A., Spivey, E. C. and Meier, K. E. (1994) J. Biol. Chem. **269**, 23790–23799
- Wang, X., Xu, L. and Zheng, L. (1994) J. Biol. Chem. **269**, 20312–20317
- Okamura, S., and Yamashita, S. (1994) J. Biol. Chem. **269**, 31207–31213
- Wang, P., Anthes, J. C., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) J. Biol. Chem. **266**, 14877–14880
- Song, J. and Foster, D. A. (1993) Biochem. J. **294**, 711–717
- Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) Cell **75**, 1137–1144

- 15 Cockcroft, S., Thomase, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) *Science* **263**, 523–526
- 16 Bowman, E, Uhlinger, DJ. and Lambeth, JD (1993) *J. Biol. Chem.* **268**, 21509–21512
- 17 Malcolm, K. C., Ross, A. H., Qiu, R. G., Symons, M. and Exton, J. H. (1994) *J. Biol. Chem.* **269**, 25951–25954
- 18 Carnero, A., Dolfi, F. and Lacal, J. C. (1994) *J. Cell. Biochem.* **54**, 478–486
- 19 Lopez, I., Burns, D. J. and Lambeth, J. D. (1995) *J. Biol. Chem.* **270**, 19465–19472
- 20 Ella, K. M. Dolan, J. W. and Meier, K. E. (1995) *Biochem. J.* **307**, 799–805
- 21 Ella, K. M., Meier, G. P., Bradshaw, C. D., Huffman, K. M., Spivey, E. C. and Meier, K. E. (1994) *Anal. Biochem.* **218**, 136–142
- 22 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.* **153**, 163
- 23 Sherman, F. (1991) *Methods Enzymol.* **194**, 3–21
- 24 Sprague, G. F., Jr. (1991) *Methods Enzymol.* **194**, 77–93
- 25 Kielland-Brandt, M. C. (1994) in *Molecular Genetics of Yeast: A Practical Approach* (Johnston, J. R., ed.), pp. 247–260, IRL Press, Oxford
- 26 Morlock, K. R., McLaughlin, J. J., Lin, Y.-P. and Carman, G. M. (1991) *J. Biol. Chem.* **266**, 3586–3593
- 27 Martinson, E. A., Trilivas, I. and Brown, J. H. (1990) *J. Biol. Chem.* **265**, 22282–22287
- 28 Lassegue, B., Alexander, R. W., Clark, M., Akers, M., and Griendling, K. K. (1993) *Biochem. J.* **292**, 509–517
- 29 Bocckino, S. B., Wilson, P. B. and Exton, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6210–6213
- 30 Khan, W. A., Blobe, G. C., Richards, A. L. and Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 9729–9735
- 31 Ahmed, S., Lee, J., Kozma, R., Best, A., Monfries, C. and Lim, L. (1993) *J. Biol. Chem.* **268**, 10709–10712
- 32 Randazzo, P. A., and Kahn, R. A. (1994) *J. Biol. Chem.* **269**, 10758–10763
- 33 Durieux, M. E. (1995) *Lysophosphatidate signaling: Cellular Effects and Molecular Mechanisms*, R. G. Landes Co., Austin
- 34 Moolenaar, W. H. (1995) *J. Biol. Chem.* **270**, 12949–12952
- 35 Billah, M. M., Lapetina, E. G. and Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399–5403
- 36 Thomson, F. J., and Clark, M. A. (1995) *Biochem. J.* **306**, 305–309
- 37 Dharmalingam, K. and Jayaraman, J. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1113–1118
- 38 Grossman, S., Cobley, J., Hogue, P. K., Kearney, E. B. and Singer, T. P. (1973) *Arch. Biochem. Biophys.* **158**, 744–753
- 39 Gopalan, G., Jayaraman, J. and Rajamanickam, C. (1984) *Arch. Biochem. Biophys.* **235**, 159–166
- 40 Mortimer, R. K., Schild, D., Contopoulou, C. R., and Kans, J. A. (1989) *Yeast* **5**, 321–403
- 41 Errede, B. and Levin, D. E. (1993) *Curr. Opin. Cell Biol.* **5**, 254–260