Identification of a Novel Ca²⁺-dependent, Phosphatidylethanolamine-hydrolyzing Phospholipase D in Yeast Bearing a Disruption in PLD¹

(Received for publication, October 7, 1996, and in revised form, November 1, 1996)

Michal Waksman‡, Xiaoqing Tang‡, Yona Eli‡, Jeffrey E. Gerst*,§, and Mordechai Liscovitch‡
From the ‡Department of Biological Regulation and §Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

We have previously reported the identification and partial characterization of a gene encoding a phospholipase D activity (PLD1) in the yeast, Saccharomyces cerevisiae. Here we report the existence of a second phospholipase D activity, designated PLD2, in yeast cells bearing disruption at the PLD1 locus. PLD2 is a Ca²⁺-dependent enzyme which preferentially utilizes phosphatidylethanolamine over phosphatidylcholine as a substrate. In contrast to PLD1, the activity of PLD2 is insensitive to phosphatidylinositol 4,5-bisphosphate, and the enzyme is incapable of catalyzing the transphosphatidylation reaction with short chain alcohols as acceptors. Subcellular fractionation shows that PLD2 localizes mainly to the cytosol, but could also be detected in the particulate fraction. Thus, the biochemical properties of PLD2 appear to be substantially different from those of PLD1. PLD2 activity is significantly and transiently elevated upon exit of wild type yeast cells from stationary phase, suggesting that it may play a role in the initiation of mitotic cell division in yeast. In view of the significantly different properties of PLD1 and PLD2, and because the yeast genome contains PLD1 as the sole member of the recently defined PLD gene family, it may be concluded that PLD2 is structurally unrelated to PLD1. Thus, the novel PLD2 activity described herein is likely to represent the first identified member of a new PLD gene family.

Phospholipase D catalyzes the hydrolysis of phospholipids at their distal phosphodiester bond to yield phosphatidic acid (PA)¹ and a free polar head group (1). Most PLDs can utilize primary alcohols as acceptors of the phosphatidyl moiety, to yield the corresponding phosphatidylalcohols (1). In mammals, different PLD activities with various substrate specificities, activation requirements, and subcellular localizations, have been described (2). PLD activity can be rapidly activated upon receptor activation or other types of cell stimulation. Receptor activation of PLD is probably mediated by multiple factors including small GTP-binding proteins, heterotrimeric GTP-binding proteins, protein kinase C, phosphatidylinositol 4,5-bisphosphate (PIP₂), tyrosine phosphorylation, and changes in the intracellular concentration of Ca²⁺ (3, 4). Phosphatidic acid, the product of the reaction catalyzed by PLD, is likely to serve as a lipid second messenger (5) and may mediate a variety of biological responses, including mitogenesis (6) and the respiratory burst (7). In addition, an ADP-ribosylation factor (ARF)-activated phosphatidylcholine (PC)-specific PLD which was discovered recently (8, 9) has been implicated in vesicular trafficking (10). The cloning of the first plant PC-specific PLD (11) enabled the identification of additional eu-karyotic PC-specific PLDs, including a yeast PLD (12–14) and a human ARF-dependent PLD (15). The eukaryotic PC-PLD gene family shares a number of common homology domains (15–17). A short common motif is shared with certain phospholipid biosynthetic enzymes that catalyze a phosphatidyl transfer reaction, but are not PLDs (15, 16). In yeast, PLD1 has no direct homologs and is therefore the sole member of this family. This paper reports the identification of a second PLD activity in yeast and describes its biochemical properties, distribution in subcellular fractions, and activation during early growth phase.

EXPERIMENTAL PROCEDURES

Chemicals—1-Palmitoyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-phosphatidylcholine (C₆-NBD-PC), 1-acyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-phosphatidylethanolamine (C₆-NBD-PE), 1-palmitoyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-phosphatidylglycerol (C₆-NBD-PG), and 1-acyl-2-[6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-phosphatidic acid (C₆-NBD-PA) were purchased from Avanti Polar Lipids. PIP₂ was obtained from Sigma.

Yeast Strains—JC1 (Mata ade8 can1 his3 leu2 lys2 trp1 ura3) (18). PLD1ΔFS-1 (Mata ade8 can1 leu2 lys2 trp1 ura3 spoΔ1:His3) (13). W303-1B (Mata ade2-1 his3-11, 15 leu2-3, 112 ura3-1 trp1-1) (19).

Media—Wild-type yeast were maintained on synthetic complete minimal medium (SC). PLD1ΔFS-1 cells were maintained on SC drop-out medium, lacking histidine. Media were prepared essentially according to Rose et al. (20).

Phospholipase D Assays—Total cell lysates were prepared as described previously (13). C₆-NBD-PC was dissolved in water. In solubilization of C₆-NBD-PE, 1.5 mM EDTA, 5 mM M C₆-NBD-PC, 1 mM EDTA, 5 mM M C₆-NBD-PE, 1.5 mM EDTA, 5 mM M C₆-NBD-PG, 1 mM EDTA, 5 mM M C₆-NBD-PA, 1 mM EDTA, were used. For determination of substrate specificity, all substrates were solubilized in 6 mM Triton X-100. The final concentration of the detergent in the reaction was 1.2 mM.

The hydrolysis of C₆-NBD-phospholipids was monitored by the production of C₆-NBD-PA, essentially as described by Danin et al. (21). For assaying PLD1 activity, we prepared cell lysates from wild-type JC1 cells. The PLD1 reaction mixture contained 0.3 mM protein, 35 mM HEPES, pH 7.2, 150 mM NaCl, 400 μM C₆-NBD-PC, 1 mM EDTA, 5 mM EGTA, and 4 mol % PIP₂. The surface concentration of PIP₂ is ex-
pressed as a percentage of the total lipid concentration. For assaying PLD2 activity, we prepared cell lysates from either wild type or pld1Δ pld1 cells. The PLD2 reaction mixture contained 0.3 mg/ml protein, 35 mM HEPES, pH 7.2, 150 mM NaCl, either 400 μM C6-NBD-PC or 40 μM C6-NBD-PG, 1 mM EDTA, 5 mM EGTA, 7 mM CaCl2, and no PIP2. The free Ca2+ concentration in the presence of EGTA and EDTA was calculated utilizing theCalccon software (Version 4.0, for MS-DOS). For the transphosphatidyltransferase assay, 300 nm concentrations of the indicated primary alcohols were added as acceptors. The reaction mixtures were incubated at 30 °C for 30 min at final volume of 120 μl. Termination, TLC separation, and quantification of the fluorescent lipid products were conducted as described (13, 21). Activity is expressed as the mean of two duplicate samples measured in arbitrary fluorescence units. Specific activity is expressed as the PA-derived fluorescent units per μg of protein.

The identity of the fluorescent product of C6-NBD-PE hydrolysis as C6-NBD-PA was verified by TLC separation of the PLD2 reaction mixtures on Silica Gel 60 aluminum plates (Merck) with the following six solvent systems: (i) chloroform/methanol/acetic acid (50:25:8); (ii) butanol/acetic acid/water (6:1:1); (iii) acetonitrile/chloroform/methanol/acetic acid/water (30:40:10:10:5); (iv) chloroform/methanol/ammonia (65:25:5); (v) the upper phase of ethyl acetate/isooctane/acetic acid/water (65:10:15:50). In all these systems, the fluorescent phospholipid product of the PLD2 enzymatic reaction co-migrated with an authentic C6-NBD-PA standard (data not shown).

RESULTS AND DISCUSSION

We have previously reported that disruption of the PLD1 gene results in complete loss of PLD activity in Δpld1 cells (13), when measured under assay conditions which included EGTA, a Ca2+ chelator (Fig. 1, left). In subsequent experiments designed to further characterize PLD1 activity and in which EGTA was omitted from the assay, we found elevated PLD activities in both wild type and Δpld1 cell lysates (data not shown). These results suggested that yeast cells express an additional, Ca2+-dependent PLD activity, which is independent of PLD1. Indeed, addition of CaCl2 at a concentration that allowed 1 mM free Ca2+ in the presence of EGTA and EDTA resulted in a substantial increase in PLD activity which was evident in both wild type and Δpld1 cell lysates (Fig. 1, right). Total PLD activity was consistently lower in Δpld1 cell lysates, reflecting the loss of PLD activity contributed by the disrupted PLD1 gene product. Therefore, the residual Ca2+-dependent PLD activity observed in Δpld1 cell lysates represents a second PLD enzyme which we have designated PLD2. Thus, use of the yeast system has allowed us to assay the activities of PLD1 and PLD2 separately: PLD1 activity could be assayed in wild type cell lysates in the presence of EGTA (which abolishes PLD2 activity), whereas PLD2 activity could be assayed in the presence of Ca2+ in Δpld1 cell lysates (in which PLD1 activity is abolished as a consequence of gene disruption).

The substrate specificity of PLD2 was next compared with that of PLD1. This was accomplished by assaying both PLD activities with 400 μM C6-NBD-PC, C6-NBD-PG, and C6-NBD-PE in the presence of 1.2 mM Triton X-100 (Fig. 2). At a concentration of 0.5 mM, Triton X-100 inhibited PLD1 activity by 25% and activated PLD2 activity using C6-NBD-PE by 30% (data not shown). We found that the production of C6-NBD-PA was up to 75-fold higher with C6-NBD-PE as a substrate compared with C6-NBD-PC. In subsequent experiments, when Triton X-100 was used in a concentration that is below its critical micelle concentration value, PLD2 activity with C6-NBD-PE was up to 10-fold higher than with C6-NBD-PC as a substrate (data not shown). In contrast, PLD1 could not utilize C6-NBD-PE as a substrate. Similar results were reported previously for PLD1 with long chain PE (12). Furthermore, compared with PLD1, PLD2 produced 4 and 11 times as much C6-NBD-PA from hydrolyzing C6-NBD-PC and C6-NBD-PG, respectively. These data suggest that a considerable difference exists in the catalytic properties of PLD1 and PLD2. In addition, the results provide a tool by which PLD1 and PLD2 activities could be discriminated in wild type cell lysates, where C6-NBD-PE can be used as a substrate for determination of PLD2 activity exclusively, without the interference of PLD1. PLD2 activity increased when increasing concentrations of either C6-NBD-PC or C6-NBD-PE were added to the reaction mixture. Using C6-NBD-PC as a substrate, PLD2 activity reached saturation at 200 μM. In contrast, saturation could not be reached with C6-NBD-PE concentrations up to 400 μM (data not shown). C6-NBD-PE was utilized routinely as a substrate in PLD2 reactions, as it is not used by PLD1 and because of the greater activity that can be achieved with this substrate. Throughout the remainder of PLD2 characterization assay, we used both C6-NBD-PC and C6-NBD-PE. The concentration used for C6-NBD-PC (400 μM) allowed enzyme saturation, and the concentration used for C6-NBD-PE was submaximal (40 μM) in the presence of 0.25 mM Triton X-100.

There is evidence for PLD-mediated hydrolysis of PE in intact mammalian cells (22, 23). Furthermore, a cytosolic PLD activity identified in various bovine tissues exhibited some preference for PE over PC as substrate (24). The mammalian cytosolic PLD was stimulated by Ca2+ (24, 25). However, in contrast to yeast PLD2, the mammalian cytosolic PLD could efficiently catalyze a transphosphatidyltransferase reaction (24, 25).

To examine the quantitative relationship between Ca2+ con-
Identification and Characterization of a Novel PLD in Yeast

Fig. 3. Effect of increasing free Ca^{2+} concentrations on PLD2 activity. Whole-cell lysates were prepared from mid-log phase Δpld1 cultures as described under "Experimental Procedures." PLD2 was assayed in the presence of 4 mol % PIP2, with either C6-NBD-PC (open circles) or C6-NBD-PE (solid circles) as described under "Experimental Procedures." Increasing amounts of Ca^{2+} were added to standard assays to yield the indicated final concentrations of free Ca^{2+}.

Fig. 4. Time course of PLD2 activation during exit from stationary phase. PLD2 activity was determined at different stages of growth in culture. A 12-h-old stationary phase culture of wild-type W303-1B cells was diluted in fresh YPD medium to 0.6 × 10^6 cells/ml and grown at 30°C. Samples were taken at the indicated times for PLD2 activity assays (solid circles) and for cell density determination (open squares). PLD2 activity was assayed as described under "Experimental Procedures" except 20 μM C6-NBD-PE was used. The arrow indicates the approximate time of entry into the first cell division cycle.

Table I

| Fraction             | Total protein | Specific activity | Total activity |
|----------------------|---------------|-------------------|----------------|
|                      | mg | fluorescence units | fluorescence units |
| Whole-cell lysate    | 50.5 | 6.8 | 341,170 |
| 8,000 × g pellet     | 3.7 | 0.01 | 52 |
| 8,000 × g supernatant| 43.0 | 4.8 | 205,440 |
| 100,000 × g pellet   | 8.0 | 3.2 | 25,800 |
| 100,000 × g supernatant| 22.5 | 4.4 | 98,490 |

Utilization of Ca^{2+} has already been demonstrated that PLD1 activity is required for meiotic cell division in yeast (12–14). It was suggested that PLD1 may inhibit mitotic cell division (36). We decided, therefore, to check whether PLD2 activity is altered during mitotic cell division. PLD2 activity was determined at different time points after the dilution of stationary-phase (G1-arrested) wild-type cells into fresh medium (Fig. 4). It was found that PLD2 was transiently activated upon culture dilution in fresh media, both before and during the interval in which the first cell division took place. Two peaks of activation could be seen. The first peak represents rapid, transient 2-fold activation of PLD2 measured within 20 min after the transfer to fresh medium and before initiation of the first cell division. This peak declines after 40 min. The second peak of activity is utilized extensively as a specific marker of PLD activity in vitro and of PLD activation in situ (see Refs. 1 and 3). Therefore, we have studied the efficiency by which PLD2 could use primary alcohols as substrates for transphosphatidylation. Ethanol, 1-propanol, and 1-butanol were added to the PLD2 reaction mixture at a concentration of 300 mM. Interestingly, PLD2 activity of Δpld1 cell lysate produced no phosphatidylalcohols whether C6-NBD-PC or C6-NBD-PE was utilized as substrate (data not shown). Thus, PLD2 appears to be incapable of transphosphatidylation when simple short chain primary alcohols are added as acceptors. This contrasts sharply with the ability of yeast PLD1 to catalyze transphosphatidylation (12–14) and with the efficient transphosphatidylation reaction carried out by the mammalian PC-hydrolyzing enzymes. Our data imply that certain forms of PLD lack this property. This conclusion is supported by previous reports showing that some PLD activities isolated from variant strains of the bacterium Streptomyces chromofuscus exhibit a greatly reduced or lack of ability to produce phosphatidylalcohols (29–31). Thus, whereas the production of phosphatidylalcohols (with simple short chain alcohols) remains a unique property of PLDs, not all PLDs can catalyze a transphosphatidylation.

Mammalian cells contain both cytosolic (24, 25, 32, 33) and membrane-bound (34, 35) forms of PLD. To examine the subcellular localization of PLD2, its activity was determined in soluble and particulate fractions derived from yeast cell lysates (Table I). Subcellular fractionation shows that most of the PLD2 activity was soluble and found in the 100,000 × g supernatant. However, a significant amount of PLD2 activity was membrane-associated.

It has already been demonstrated that PLD1 activity is required for meiotic cell division in yeast (12–14). It was suggested that PLD1 may inhibit mitotic cell division (36). We decided, therefore, to check whether PLD2 activity is altered during mitotic cell division. PLD2 activity was determined at different time points after the dilution of stationary-phase (G1-arrested) wild-type cells into fresh medium (Fig. 4). It was found that PLD2 was transiently activated upon culture dilution in fresh media, both before and during the interval in which the first cell division took place. Two peaks of activation could be seen. The first peak represents rapid, transient 2-fold activation of PLD2 measured within 20 min after the transfer to fresh medium and before initiation of the first cell division. This peak declines after 40 min. The second peak of activity is...
observed 2–4 h after the transfer and then declines. These results suggest a biphasic response of PLD2 to induction of mitotic cell division in G1–arrested cells and indicate that PLD2 is a regulated enzyme. Interestingly, this activation of PLD2 was detected under in vitro assay conditions. Hence, the stimulation of PLD2 activity during early growth phase may involve a persistent modification of the enzyme. The rapid activation upon growth stimulation suggests that PLD2 may mediate early growth signals and play a role in the initiation of the mitotic cell cycle. In mammalian cells, PLD is activated by growth factors and other mitogens, suggesting a role in mediating G0-G1 transition (6). In addition, there is evidence for a negative modulatory effect of PLD also on G2-M phase transition (37). Presumably, the PA produced by PLD2 may regulate one or more of the proteins involved in cell cycle control.

The radically different catalytic properties of PLD1 and PLD2 indicate that these two enzymes are probably structurally unrelated. Indeed, an extensive analysis of the complete S. cerevisiae genome by the BLAST algorithm, using several highly conserved domains present in known PLD family members as well as the putative Ca²⁺-binding domain of plant PLD, has demonstrated that PLD1 has no direct homologs. Thus, in yeast, PLD1 is the sole member of the recently defined gene family (15, 16) that, in addition to PLD1, includes the plant PLDs, the human ARF-dependent PLD, and a putative Cae

norhabditis elegans PLD gene. Consequently, we postulate that the gene encoding PLD2 does not belong to the PLD/phosphatidyltransferase gene family and, thus, may represent the first identified member of a novel PLD family. Ongoing work in our laboratory is aimed at identifying and cloning the PLD2 gene.

REFERENCES

1. Heller, M. (1978) in Advances in Lipid Research (Paoletty, R., and Krizhevsky, D., eds) pp. 267–326, Academic Press, New York

2. Liscovitch, M., and Chalifa-Caspi, V. (1996) Chem Phys Lipids 80, 37–44

3. Liscovitch, M., and Chalifa, V. (1994) in Signal-activated Phospholipases (Liscovitch, M., ed) pp. 31–63, R. G. Landes Co., Austin

4. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42

5. English, D., Cui, Y., and Siddiqui, R. A. (1996) Chem. Phys. Lipids 80, 117–132

6. Boarder, M. R. (1994) Trends Pharmacol. Sci. 15, 57–62

7. McPhail, L. C., Quailiotine-Mann, D., Agwu, D. E., and McCall, C. E. (1993) Eur. J. Haematol. 51, 294–300

8. Brown, H. A., Gutowski, S., Moowam, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144

9. Cockcroft, S., Thomas, G. 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

10. Cockcroft, S. (1996) Chem. Phys. Lipids 80, 59–80

11. Wang, X., Xu, L., and Zheng, L. (1994) J. Biol. Chem. 269, 20312–20317

12. Rose, K., Rudge, S. A., Frohman, M. A., Morris, A. J., and Engebrecht, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12151–12155

13. Waksman, M., Eli, Y., Liscovitch, M., and Gerst, J. E. (1996) J. Biol. Chem. 271, 2981–2986

14. Ella, K., Dolan, J. W., Qi, C., and Meier, K. E. (1996) Biochem. J. 314, 15–19

15. 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

16. Ponting, C. P., and Kerr, I. D. (1996) Protein Sci. 5, 914–922

17. Ribbes, G., Henry, J., Carivien, C., Pontarotti, P., Chap, H., and Record, M. (1996) Biochem. Biophys. Res. Commun. 226, 208–211

18. Field, J., Vojtek, A., Ballester, R., Bolger, G., Colicielli, J., Ferguson, K., Gerst, J., Kataoka, T., Michaeli, T., Powers, S., Riggs, M., Rodgers, L., Wieland, I., Whelan, B., and Wigler, M. (1995) Cell 61, 319–327

19. Ackerman, S. H., and Tzagoloff, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4986–4990

20. Rose, M. D., Winston, F., and Hieter, P. (1996) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

21. Danin, M., Chalifa, V., Mohn, H., Schmidt, U. S., and Liscovitch, M. (1993) in Lipid Metabolism in Signaling Systems (Fain, J. N., ed) pp. 14–24, Academic Press, San Diego

22. Kies, Z. (1992) Biochem. J. 285, 229–233

23. Kies, Z. (1996) Chem. Phys. Lipids 80, 81–102

24. Wang, P., Anthes, J. C., Siegel, I. M., Egan, R. W., and Billah, M. M. (1991) J. Biol. Chem. 266, 14877–14880

25. Siddiqui, A. R., Smith, J. L., Ross, A. H., Qi, R.-G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem. 270, 8466–8473

26. Perridge, M. J. (1995) Nature 371, 315–325

27. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S., and Cantley, L. C. (1994) J. Biol. Chem. 269, 21403–21406

28. Massenburg, D., Han, J.-S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J., and Vaughan, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11718–11722

29. Juneja, L. R., Kazuoka, T., Michaeli, T., Powers, S., Riggs, M., and Engebrecht, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9761–9765

30. Kies, Z. (1996) Biochim. Biophys. Acta 960, 334–341

31. Ghosh, S. S., and Franson, R. C. (1992) Biochim. Cell Biol. 70, 43–48

32. Balsinde, J., Diez, E., Fernandez, B., and Molinero, F. (1989) Eur. J. Biochem. 186, 717–724

33. Huang, C., Wykle, R. L., Daniel, L., and Cabot, M. C. (1992) J. Biol. Chem. 267, 16859–16865

34. Taki, T., and Kanfer, J. N. (1979) J. Biol. Chem. 254, 14935–14943

35. Brown, H. A., Gutowski, S., Kohn, R. A., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14935–14943

36. Honigberg, S., and Esposito, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6550–6563

37. Kaszkin, M., Richards, J., and Kinzel, V. (1996) Biochem. J. 314, 129–138
Identification of a Novel Ca^{2+}-dependent, Phosphatidylethanolamine-hydrolyzing Phospholipase D in Yeast Bearing a Disruption in PLD1
Michal Waksman, Xiaoqing Tang, Yona Eli, Jeffrey E. Gerst and Mordechai Liscovitch

J. Biol. Chem. 1997, 272:36-39. doi: 10.1074/jbc.272.1.36

Access the most updated version of this article at http://www.jbc.org/content/272/1/36

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 16 of which can be accessed free at http://www.jbc.org/content/272/1/36.full.html#ref-list-1