Characterization of a regulated form of phospholipase D in the yeast *Saccharomyces cerevisiae*

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Phospholipase D (PLD), which is present in bacterial, plant and animal cells, can serve as an important element of signal-transduction pathways. This study examined the potential role of this enzyme in the regulation of *Saccharomyces cerevisiae*. An assay *in vitro* using a fluorescent 1-acyl-2-alkyl glycerophosphocholine as substrate was used to assess PLD activity in yeast cell extracts. A neutral PLD activity is present in membranes prepared from both haploid and diploid yeast cells, as evidenced by the production of phosphatidic acid and phosphatidylbutanol in the presence of butanol. Alcohols, in addition to serving as substrates for transphosphatidylation, stimulate PLD activity. Increased PLD activity is detected in membranes when either haploid or diploid cells are incubated in the presence of a non-fermentable carbon source. Membrane PLD activity increases within 10 min after diploid cells are placed in a sporulation-inducing medium lacking nitrogen and containing a non-fermentable carbon source. The increased activity persists for 2–3 h, and then declines to control values. This response occurs in the presence of cycloheximide, an inhibitor of protein synthesis. These data indicate that PLD activity is present in yeast, and that activation of PLD is an early event in sporulation in this organism.

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phospholipid [predominantly phosphatidylcholine (PC)] to phosphatidic acid (PA) [1,2]. This enzyme, which is activated in mammalian cells in response to a wide variety of stimuli, may be important in mitogenic signalling [3,4]. PLD can be activated via both protein kinase C [5] and guanine-nucleotide-binding proteins [6] in mammalian cells. In some cases, PLD activation is dependent on ARF (ADP-ribosylation factor) proteins [7,8]. ARF proteins are guanine-nucleotide-binding proteins that are involved in membrane trafficking [9]. PA is a putative second messenger that can affect the activities of protein kinases [10], guanine-nucleotide-binding proteins [11,12] and phospholipase C-γ1 [13]. PA also serves as a substrate for the production of other messenger molecules: diacylglycerol (DG), arachidonic acid, and lyso-phosphatidic acid (lyso-PA). Lyso-PA binds to receptors linked to activation of phospholipase C (PLC) and PLD [14]. Transphosphatidylation, a reaction in which alcohols serve as substrates for the production of phosphatidylalcohols, is catalysed by plant and mammalian PLDs. PLD can catalyse the formation of bisphosphatidic acid, another putative second messenger molecule, in the presence of DG [15].

Since only one eukaryotic PLD has been purified or sequenced, it is not clear how many isoforms of the enzyme exist. More than one form appears to be activated in response to extracellular signals [16]. Recent evidence suggests that at least one regulated form of mammalian PLD preferentially utilizes glycerophosphocholines with a 1-alkyl linkage [16,17]. Both cytosolic and membrane forms of PLD have been identified in mammalian tissues [18]. The sequence for a plant PLD gene was recently obtained; the single polypeptide catalyses both hydrolysis and transphosphatidylation reactions [19].

In order to define further the role(s) of PLD in cellular signal transduction, we examined whether a PLD with transphosphatidylation activity was present in the yeast *Saccharomyces cerevisiae*. This organism utilizes many signalling pathways analogous to those of mammalian cells; a notable example is the involvement of mitogen-activated protein kinases in the pheromone response pathway [20–22]. The role of phospholipases in yeast signal transduction has only recently been investigated; a gene encoding a homologue to the mammalian phosphatidylinositol-specific PLC has been identified in *S. cerevisiae* [23,24]. Two forms of PA phosphohydrolase have been identified in yeast [25]. These enzymes are regulated by growth phase [24], phosphorylation [26], and sphingoid bases [27]. PLD activity was detected in yeast mitochondria, although its transphosphatidylation capability was not studied [28–30]. This PLD activity was increased in mitochondria in response to glucose repression. These observations suggest that a PLD activity similar to that of mammalian cells might be present, and subject to regulation, in yeast.

In this report, we use an assay *in vitro* to demonstrate that yeast cells express PLD activity that is localized to the membrane fraction. The properties of this enzyme are described. Furthermore, our studies show that PLD is activated in response to sporulation conditions in diploid yeast, suggesting that PLD may play a role in adaptive responses in these cells.

EXPERIMENTAL

Yeast cell strains

The following yeast strains were used: EG123 (*MATa can1-101 his4-519 leu2 trp1-1 ura3*), 246-1-1 (*MATα can1-101 his4-519*

Abbreviations used: AAG, 1-acyl-2-alkyl-glycerol; ARF, ADP-ribosylation factor; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; B-HPC, diacyl-2-BODIPY-phosphatidylcholine; BPA, BODIPY-phosphatidic acid; BPC, 1-acyl-2-alkyl-1-BODIPY-glycerophosphocholine; B-SM, BODIPY-sphingomyelin; DG, diacylglycerol; PA, phosphatidic acid; PAF, platelet-activating factor; PBt, phosphatidylbutanol; PC, phosphatidylcholine; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; YEP, yeast extract peptone.

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leu2 trp1-1 ura3), AM227α (*MATα lys1*), and EG123/AM227α (diploid derived by mating EG123 with AM227α).

Preparation of cell extracts

Yeast cells were grown in the following media: YPD [yeast extract peptone (YEP) containing 2% (w/v) glucose], YPEG (YEP containing 2% glycerol and 2% ethanol), YPA (YEP containing 2% potassium acetate), YPGal (YEP containing 3% galactose), or SC (synthetic complete medium; 2% glucose). YEP and SC were prepared as described previously [31]. For experiments involving sporulation, diploid cells were grown for 1 day in YPD medium, then switched to YPA medium for 20 h. The cells were washed with water, and then resuspended in 1% potassium acetate. After 24 h, the spores were washed with water and then resuspended in YPD medium to induce germination. Cell pellets, collected at various times during this procedure, were frozen at −20 °C before disruption.

Yeast cell pellets were disrupted by vortex-mixing with glass beads in lysis buffer (20 mM Hepes, 80 mM β-glycerophosphate, 10 mM EGTA, 2 mM EDTA, 25 μM leupeptin, 10 μg/ml aprotinin, 2 mM dithiothreitol, pH 7.5) at 4 °C. Beads and intact cells were removed by centrifugation in a micro-centrifuge for 15 s. Cytosolic and membrane extracts were obtained after centrifugation of the cell lysates at 100000 **g** for 20 min at 4 °C. For some experiments, the extracts were stored at −70 °C before assay.

PLD assays

PLD assays were conducted *in vitro* with a BODIPY-glycerophosphocholine (BPC; 2-decanoyl-1-{*O*-[(11-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-propionyl)amino]-undecyl}-*sn*-glycero-3-phosphocholine) as substrate, as described previously [32]. BPC (BODIPY-FL C_3C_{11}-C_{10}-PAF, catalogue no. D-3771), BODIPY-HPC [2-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; β-BODIPY FL C_{12}-HPC, catalogue no. D-3792] and BODIPY-sphingomyelin [B-SM; *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-pentanoyl)sphingosylphosphocholine; β-BODIPY FL sphingomyelin, catalogue no. D-3522] were obtained from Molecular Probes. BODIPY (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene) is a trademark of Molecular Probes. For some experiments, BODIPY-phosphatidic acid [BPA; 2-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate; β-BODIPY-FL C_{12}-HPA, Molecular Probes, catalogue no. D-3801] was used as substrate. A sample of 1 mM BPC or BPA in ethanol was dried under nitrogen and briefly sonicated in 500 μM octylglucoside/400 mM NaCl/66 mM Hepes (pH 7.0) before use. The final reaction (12.5 μl) contained 0.1 mM BPC, 150 mM NaCl, 200 μM octylglucoside, 25 mM Hepes (pH 7.0), 5 mM EGTA, 1 mM EDTA, 40 mM β-glycerophosphate, 1 mM dithiothreitol and 1% (v/v; 0.135 M) n-butanol. The reaction, initiated by addition of 5 or 10 μg of cell protein, was allowed to proceed for 40 min at 30 °C. For some experiments, partially purified phospholipases were used: PLD (0.5 μg/μl) from cabbage (Sigma) and PLC (0.5 unit/μl) from *Clostridium perfringens* (type XIV, Sigma). DL-propranolol and dipalmitoyl-phosphatidylcholine were from Sigma. C_{16}-PAF (platelet-activating factor; 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and other PAF analogues were from Cayman Chemicals. A 5 μl sample of the reaction mixture was applied to a plastic-backed silica gel G60 plate without fluorescent indicator (Merck); reaction products were separated by development in chloroform/methanol/water/acetic acid (45:45:10:1, by vol.). TLC plates were photographed under long-wave UV light by using TechPan Film (Kodak). The results were quantified by using a Cliniscan 2 fluorescence scanning densitometer (Helena Laboratories) at 507 nm excitation and 514 nm emission. For some experiments, results were imaged and quantified by using a FluorImager (Molecular Dynamics). Fluorescence in a single band was quantified as a percentage of total fluorescence observed for each reaction (entire lane of the TLC plate) to correct for any variations in sample application. This value was then converted into pmol/min per mg of protein. Protein determinations were performed by a Coomassie-Blue binding assay (Pierce Chemical Co.) with bovine serum albumin standards.

RESULTS

Characterization of PLD in yeast

In order to identify PLD activity in *S. cerevisiae*, we used an assay *in vitro* for PLD activity. This assay, which utilizes a fluorescent glycerophosphocholine as substrate, can be used to detect the activated state of PLD in membranes prepared from agonist-treated mammalian cells [32]. An alcohol is routinely included in PLD assays to allow formation of a phosphatidylalcohol by the transphosphatidylation reaction that is unique to PLD. The phosphatidylalcohol product is less susceptible to further hydrolysis than is PA. Membrane and cytosolic extracts prepared from yeast cells were tested for PLD activity. PLD activity, as assessed by the formation of PA and phosphatidylbutanol (PBt), was present primarily in the membrane fractions (Figure 1). The activity was present in similar amounts in membrane fractions prepared from both haploid (**a** or α strains) and diploid (**a**/α) yeast cells. The enzyme activity was greatest at pH 7.0–7.5 (results not shown). These results demonstrate that yeast cells express a neutral PLD activity that is localized to the cell membrane.

Further details concerning the PLD reaction conditions are as follows (results not shown). NaCl was required for activity, under these conditions, whereas EGTA, EDTA, β-glycerophosphate and dithiothreitol (all components of the cell-lysis buffer) were not required. The substrate (BPC) was present at saturating concentrations. The assay results were linear with protein concentrations up to 15 μg/reaction and at incubation times up to 60 min. Since stimulated enzyme activity (see below) was sensitive to freeze–thawing, PLD activity was usually measured on the same day that the extracts were prepared.

Yeast cell membranes contain additional enzymes that utilize BPC as a substrate. The production of lyso-PC indicates the presence of a glycerophosphocholine-utilizing phospholipase A_2 (PLA_2). The 1-acyl-2-alkyl-glycerol (AAG) seen in Figure 1 could be generated via hydrolysis of the substrate by a glycerophosphocholine-utilizing PLC, or could reflect metabolism of PA to AAG. In the solvent system used here, BPC and PA are well resolved, while AAG and monoacylglycerol co-migrate. When an alternative solvent system (chloroform/methanol/acetic acid, 13:3:1, by vol.) was used that was capable of resolving AAG and monoacylglycerol, the results showed that only AAG was produced in the reaction (results not shown).

In order to determine whether the AAG produced in the reactions with yeast membrane preparations was derived from PA, BODIPY-phosphatidic acid (BPA) was used as a substrate under the same conditions used for the PLD reaction. Approx. 30% of the BPA was converted into AAG in 40 min (Figure 2). Thus yeast membranes contain a PA phosphohydrolase activity that is capable of producing AAG from PA, resulting in some under-estimation of PLD activity. Propranolol, at high concen-

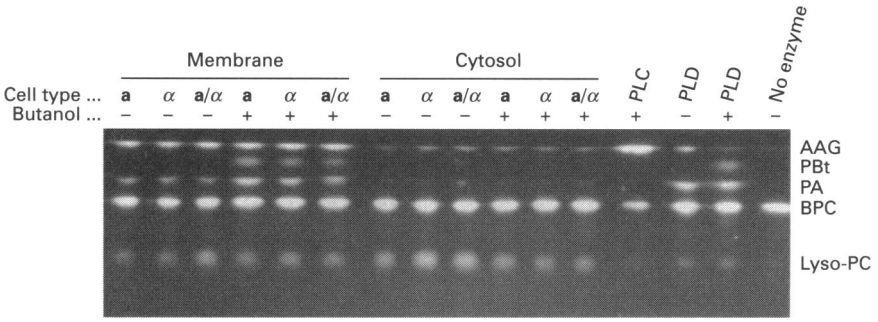

Figure 1 PLD activity in haploid and diploid yeast

Membrane and cytosolic fractions were prepared from α and **a** haploid cells and from **a**/α diploid cells as described in the Experimental section. These fractions were assayed for PLD activity in the absence and presence of 0.5% butanol, using BPC as substrate. The reaction was carried out as described in the Experimental section. Reactions were also carried out with commercially obtained preparations of PLC and PLD and in the absence of enzyme. The reaction products were separated by TLC, and photographed under UV light.

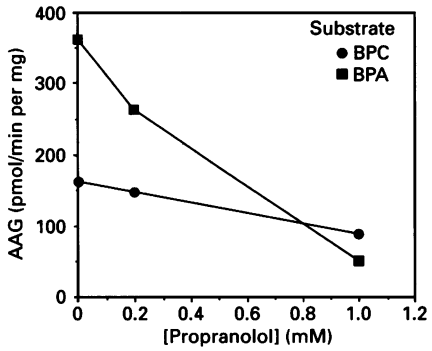

Figure 2 PA phosphohydrolase activity in yeast membranes

Membranes prepared from diploid cells were incubated with either BPC or BPA, as described in the Experimental section, in the presence of the indicated concentrations of propranolol. Production of AAG is shown.

trations, has been reported to inhibit PA phosphohydrolase in intact mammalian cells [33,34] as well as in extracts prepared from these cells [35]. Production of AAG from either BPC or BPA by yeast membranes was inhibited in the presence of propranolol (Figure 2). These results indicate that a portion of the AAG is derived from the sequential actions of PLD and a PA phosphohydrolase.

The results shown in Figure 1 suggest that the yeast enzyme(s) does not utilize alcohol efficiently in the transphosphatidylation reaction, since substantial amounts of PA were present in addition to PBt. The effects of various alcohols on the PLD reaction were examined (Table 1). The ability of the straight-chain alcohols to serve as substrates varied with chain length, with maximal effects seen with pentanol. The decreased utilization of longer-chain alcohols may, in part, be due to their low solubility in water. 3-Methylbutan-1-ol (isoamyl alcohol), a branched-chain alcohol, served as a substrate for the PLD reaction. In addition, it was clear that alcohols that could be utilized as substrates had a stimulatory effect on PLD activity, as seen by the increase in the sum of PA and phosphatidyl-alcohols. The rank order for the effects of straight- and branched-chain alcohols of varying chain length on PLD activity was similar to that seen with plant and mammalian cell PLDs (K. M. Ella, K. E. Meier, A. Kumar, Y. Zhang and G. P. Meier, unpublished work). These results indicate

Table 1 Effects of alcohols on PLD activity from yeast membranes

Membranes prepared from diploid cells were assayed for PLD activity as described in the Experimental section. Alcohols of different chain lengths were added to the reaction mixture at a final concentration of 0.135 M. Abbreviation: POR, phosphatidyl-alcohol.

	PLD activity (pmol/min per mg)		
Alcohol	PA	POR	PA + POR
None	75.0	0	75.0
Straight-chain			
Methanol	37.5	18.8	56.3
Ethanol	45.0	21.3	66.3
Propanol	53.8	27.5	81.3
Butanol	60.0	50.0	110.0
Pentanol	97.5	50.0	147.5
Hexanol	81.3	31.9	113.2
Heptanol	81.9	31.3	113.2
Octanol	67.5	0	67.5
Branched-chain			
Propan-2-ol	58.8	0	58.8
2-Methylpropan-1-ol (isobutyl)	63.8	22.5	86.3
3-Methylbutan-1-ol (isoamyl)	115.6	46.9	162.5
Butan-2-ol	62.5	0	62.5
2-Methylpropan-2-ol (t-butyl)	53.8	0	53.8
2,2-Dimethylpropan-1-ol (neo-pentanol)	46.9	0	46.9

that the PLD activity present in yeast cell membranes possesses an active site similar to those present in PLDs from plant and mammalian cells.

The ability of alcohol to stimulate the PLD reaction was studied in more detail. In Figure 3, the dose–response relationship for the effects of ethanol are shown. Maximal levels of both PA and phosphatidylethanol, the transphosphatidylation product, were observed with approx. 250 mM ethanol. Similar results were obtained in experiments using butanol, i.e. the concentration of butanol giving maximal transphosphatidylation product also gave maximal production of PA (results not shown). Taken together, the results shown in Figure 3 and Table 1 suggest that the utilization of alcohols as substrates for transphosphatidylation is associated with an increase in enzyme activity. Similar results have been observed in our laboratory for plant and

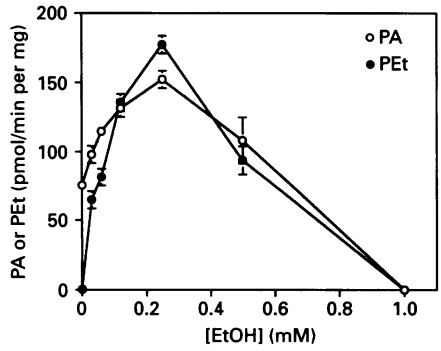

Figure 3 Effects of ethanol on PLD activity from yeast membranes

Membranes prepared from diploid cells were assayed for PLD activity in the presence of the indicated concentrations of ethanol, as described in the Experimental section. Production of PA and phosphatidylethanol (PEt) is shown. Each point represents the mean ± S.D. of values obtained from duplicate experiments.

Table 2 Utilization of phospholipid substrates by yeast PLD

Membranes prepared from diploid cells were assayed for PLD activity in the presence of 0.1 mM concentrations of the indicated substrates. In Expt. A, membranes were incubated with BPC, B-HPC or B-SM alone, with both BPC and B-HPC or B-SM, or with both B-HPC and B-SM. In Expt. B, membranes were incubated with 0.1 mM BPC in the absence or presence of equimolar concentrations of dipalmitoyl-PC (PC), 1-acyl-2-alkyl-2-C_{16}-glycerophosphocholine (C_{16}-PAF), 1-acyl-2-alkyl-2-C_{18}-glycerophosphocholine (C_{18}-PAF), 1-alkyl-2-acyl-2-arachidonyl-glycerophosphocholine (A-PAF), or 1-acyl-2-alkyl-2-*O*-methyl-glycerophosphocholine (OMePAF). Combined production of PA and PBt is shown. Each point represents the mean ± S.D. of values obtained from duplicate experiments.

Substrate	PA + PBt (pmol/min per mg)
Expt. A	
B-PC	123.8 ± 0
B-HPC	0 ± 0
B-PC + B-HPC	101.6 ± 0.9
B-SM	0 ± 0
B-PC + B-SM	114.4 ± 2.7
Expt. B	
B-PC	118.8 ± 8.8
B-PC + PC	98.1 ± 2.7
B-PC + C_{16}-PAF	111.3 ± 7.1
B-PC + C_{18}-PAF	118.1 ± 2.7
B-PC + A-PAF	121.3 ± 1.8
B-PC + OMe-PAF	111.9 ± 2.7

mammalian forms of PLD (K. M. Ella, K. E. Meier, A. Kumar, Y. Zhang and G. P. Meier, unpublished work).

The utilization of phospholipid substrates by the yeast PLD was investigated (Table 2). Two other fluorescent substrates, B-HPC (a diacyl-2-BODIPY-PC) and B-SM, did not serve as substrates for the transphosphatidylation reaction. B-HPC inhibited utilization of BPC by approx. 15%, suggesting that it was capable of interacting with PLD. The failure of B-HPC to serve as a substrate may be due to conjugation of the bulky fluorophore moiety to the fatty acid located at the 2-position of the glycerol backbone, or could be due to the presence of an acyl linkage at the 1-position. The abilities of non-fluorescent phospholipids to compete for utilization of BPC were examined. Dipalmitoyl-PC inhibited utilization of BPC by approx. 20% when present at equimolar concentrations with BPC. C_{16}-PAF, an 1-acyl-2-alkyl-glycerophosphocholine with palmitic acid at the 2-position, was slightly less effective. PAFs with oleic or arachidonic acid at the 2-position, or 2-*O*-methyl-PAF, did not significantly interfere with hydrolysis of BPC. Interpretation of the competition results is complicated by the presence of unknown concentrations of endogenous phospholipids in the solubilized membranes, and by the observation that B-HPC can inhibit utilization of BPC without serving as a substrate. These problems are reflected by the observation that 2-fold higher concentrations of the competing lipids did not give significantly greater competition (results not shown). In mammalian cell membranes, PC utilization is maximal only within a narrow concentration range, with decreased activity observed with higher concentrations of substrate [18]. Nonetheless, these results suggest that BPC is a favoured substrate for yeast PLD. Since the results do not establish that the enzyme prefers alkyl-linked glycerophosphocholines, other features of BPC (including the presence of the BODIPY fluorophore at the 1-position of the glycerol backbone) may contribute to its preferential utilization by the enzyme.

Regulation of PLD in yeast cells

Further experiments were directed at determining whether PLD activity is regulated in yeast cells in response to extracellular stimuli. As shown in Figure 1, similar levels of activity were observed in both haploid and diploid cells. PLD activity was also similar in stationary and in growing cells (results not shown). The response of haploid yeast cells to pheromone is mediated via activation of mitogen-activated protein kinases [20,21]. This signalling pathway is analogous to that seen in mammalian cells exposed to growth factors. However, PLD activity was not altered in response to treatment of **a** cells with α-pheromone (results not shown). These results prompted us to examine whether PLD activity was altered in response to other regulators of yeast cell function.

Yeast cells are regulated by nutritional status. Cells are able to grow by fermenting carbon sources such as glucose, or by respiring non-fermentable carbon sources such as glycerol and acetate. To determine whether PLD activity was regulated in response to growth in different carbon sources, PLD activity was measured in membranes from cells grown with either YPD (glucose) or YPA (acetate). Growth on acetate resulted in a consistent, albeit small, increase in PLD activity in both haploid and diploid cells. Values for combined production of PA and PBt were increased by 19.7 ± 0.02% (mean ± S.D. of duplicate experiments) in haploid cells grown in YPA as compared with YPD. In diploid cells, these values were 23.8 ± 0.08% higher in YPA than in YPD. Growth of haploid cells in SC medium or galactose resulted in levels of PLD activity intermediate between those seen with YPD and YPA (results not shown). These results suggest that PLD activity is regulated in response to changes in carbon source.

The effect of acetate on PLD activity is interesting in light of the fact that acetate is the most effective non-fermentable carbon source in terms of its ability to induce sporulation of diploid cells [36]. Sporulation is induced by depriving cells of a nitrogen source in the presence of a non-fermentable carbon source. PLD activity was assayed at various times after suspension of diploid cells in sporulation medium (1% acetate) (Figure 4a). Switching cells from rich medium containing acetate (YPA) to 1% acetate resulted in a rapid increase in PLD activity, detectable within the

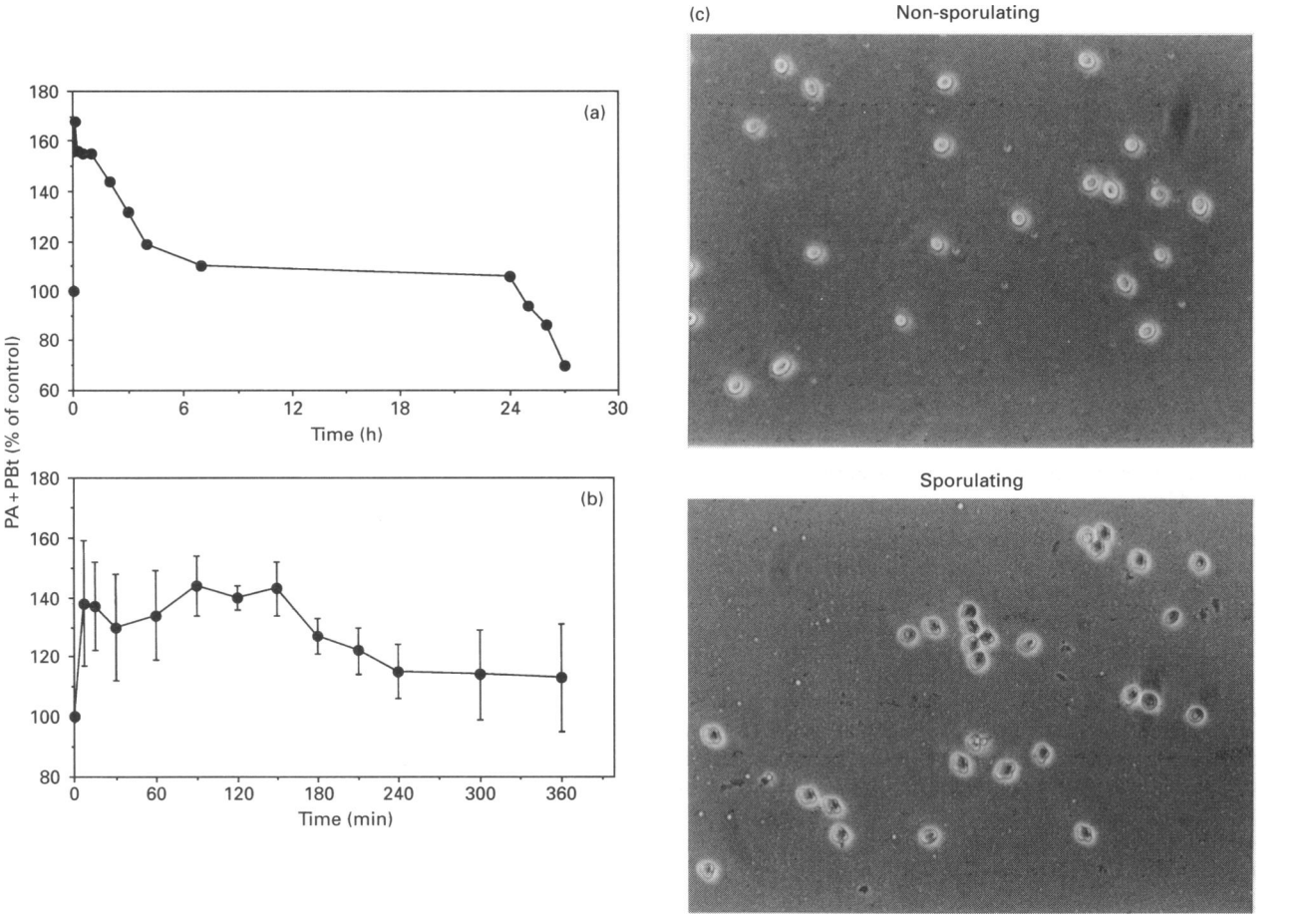

Figure 4 Effects of sporulation on PLD activity in yeast cell membranes

In (**a**) sporulation was induced by incubating diploid cells (grown in YPA medium) in the presence of 1% acetate. PLD activity was assessed in membranes prepared from diploid cells at various times after transfer to 1% acetate. PLD activity was also measured at the indicated times after transfer of the sporulated cells to rich (YPD) medium, inducing germination. In (**b**), the time course of the effects of sporulation on PLD activity is shown as the mean ± S.D. of results from three separate experiments using different diploid cells. For both panels, PLD activity (PA + PBt) is expressed as a percentage of the control value measured before transfer to 1% acetate or YPD. In (**c**), non-sporulating (grown in YPA) and sporulating (grown in 1% acetate) diploid cells were photographed using phase-contrast microscopy. Magnification × 150.

first 10 min. The following results (not shown) exclude an effect of sporulation on other BPC- or PA-metabolizing enzymes. Changes in the activities of PLA_2s utilizing BPC or PBt as substrates were not apparent, as there were no significant changes in the production of lyso-PC, and lyso-PBt was not observed as a product with yeast membranes. DG production was slightly increased after nutrient deprivation, as would be expected, due to the increased availability of substrate for PA phosphohydrolase. DG kinase activity was not detected in this reaction, which was run in the absence of ATP. The increased production of PBt was paralleled by increased production of PA. The increase in PLD activity was similar when 200 μM propranolol was included in the assay, although the absolute levels of PA were slightly higher (results not shown). These results indicate that the observed changes in activity do not reflect changes in product metabolism.

The time course of the activation of PLD in response to nutrient deprivation is of interest. The level of activity remained elevated for the first 2–3 h and then returned to initial values by 24 h. Since the initial level represents activity already induced by growth on acetate, the level of activity after 24 h was still greater than that seen with cells grown on glucose. Suspending the spores in rich glucose-containing medium (YPD) led to germination and a rapid decrease in PLD activity to a level comparable with that seen in diploid cells growing vegetatively on glucose. The results in Figure 4(a) are from a single experiment. Each experiment was performed with freshly mated diploid cells to enhance the synchrony and extent of sporulation. In Figure 4(b), the results of three independent experiments have been combined. Since different diploids were used in each experiment, the exact timing and absolute extent of induction of PLD activity varied somewhat between experiments. Nonetheless, it is clear that PLD activity is greatest when diploid cells are suspended in acetate and begin to sporulate. In these experiments, 80–95% of the cells had sporulated after 24 h in sporulation medium (Figure 4c). If cells are suspended in sporulation medium and then transferred back to rich medium within the first several hours, the cells will return to vegetative growth and will not sporulate [37]. Once the cells have passed this critical commitment step, they will continue to undergo meiosis and sporulation regardless of the presence of rich medium. The results shown here indicate that maximal PLD activity persists through much of the period during which cells become committed to sporulation.

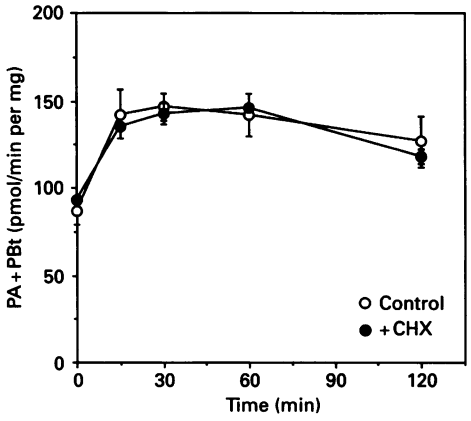

Figure 5 Effects of cycloheximide on PLD activation in response to sporulation medium

PLD activity was assessed in membranes prepared from diploid cells at various times after transfer to 1% acetate. Cycloheximide (CHX; 10 μg/ml) was added at the time of the change of medium. Each point represents the mean ± S.D. of values obtained from duplicate samples of cells that were incubated in parallel.

In order to examine whether the increase in PLD activity occurred as a result of protein synthesis, cells were switched to sporulation medium in the presence of cycloheximide. As shown in Figure 5, cycloheximide had no effect on the increase in PLD activity. Therefore, the observed response apparently reflects activation, rather than increased expression, of the enzyme.

DISCUSSION

The results presented here demonstrate that yeast cells express a PLD activity that can utilize alcohols as substrates. This activity is present in all yeast cell types, but can be regulated in both haploid and diploid cells in response to nutrients. The PLD activity previously identified in yeast was mitochondrial. Mitochondrial membranes are probably present in the membrane fraction used in our studies. However, in contrast with previously published results [30], our results indicate that PLD activity is not increased in response to glucose repression. This difference in responsiveness may suggest that more than one form of PLD is present in yeast.

Our data indicate that PLD activation can serve as an early biochemical marker for the induction of sporulation. The role of PA as a signalling molecule in these events remains to be determined. As is postulated for mammalian cells, PA may itself serve as a second messenger. Alternatively, PA may serve as a major source for DG production in yeast cells (Figure 1). The regulation of yeast PA phosphohydrolase [27] may play an important role in these adaptative responses. The DG generated by this pathway may serve to activate yeast forms of protein kinase C [38]. The phospholipid utilized as substrate for the PLD reaction in intact cells may be important. For these studies, an alkyl-linked form of PC was utilized as substrate. This form of PC appears to be the preferred substrate for at least one form of phorbol-ester-activated mammalian PLD [16,17]. However, alkyl-linked phospholipids have not yet been detected in yeast. Other properties of the yeast enzyme, particularly in regard to the effects of alcohols, appear to be similar to those of plant and animal forms of the enzyme. It is noteworthy that ethanol, a fermentation product of yeast cells, can stimulate PLD activity. Additional analyses of phospholipid hydrolysis in intact yeast cells will be required to put these observations into perspective.

Control of sporulation is an extremely complex process that incorporates multiple pathways that sense the ploidy of the cell and the nutritional status. A key regulator of meiosis and sporulation is the product of the *IME1* gene [39]. This gene is regulated at the transcriptional level by both mating type and nutritional state, and may represent the convergence point for signals generated by these two stimuli. Previous studies have implicated the GTP-binding protein *RAS2* [40] and adenylate cyclase *CYR1* [41] in the nutritional control of sporulation. It remains to be determined whether PLD activity is regulated in a *RAS2*-dependent manner. In mammalian neutrophils and liver, activation of PLD is dependent on the activity of *rho* proteins, which are members of the *ras* family [42,43].

Recent studies have focused on the role of guanine-nucleotide-binding proteins, including ARF, as both regulators and effectors for PLD. Yeast expresses two forms of ARF [44]. Null mutations in both genes are lethal. Although it remains to be determined whether yeast PLD is regulated by guanine-nucleotide-binding proteins, it is likely that analogous pathways for PLD activation exist in yeast and mammalian cells.

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