Phospholipase D activity is required for dimorphic transition in Candida albicans

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Candida albicans is an opportunistic pathogen that causes significant morbidity and mortality in immunocompromised patients. In this report, the presence of a phospholipase D (PLD) activity in C. albicans, designated CaPLD1, is demonstrated. This is the first description of PLD activity in this organism. CaPLD1 activity was stimulated by inducers of dimorphic transition. Furthermore, transition was stimulated by the addition of exogenous PLD to cells. The addition of 1-propanol to the medium, which resulted in the production of phosphatidylpropanol by CaPLD1 at the expense of the usual product phosphatidic acid, delayed the yeast to hypha transition. These results suggest that CaPLD1 may be an important regulator of dimorphic transition in C. albicans.

Keywords: Candida albicans, phospholipase D, dimorphic transition

INTRODUCTION

Phospholipases have emerged as significant components of numerous signal transduction pathways in a wide variety of organisms. Phospholipase D (PLD) activity has been identified in mammals, plants, bacteria (Heller, 1978) and fungi (Ella et al., 1995). The preferred substrate of PLD is the phospholipid phosphatidylcholine, which is hydrolysed to phosphatidic acid. Phosphatidic acid itself can function as a second messenger (Amsterdam et al., 1994). The formation of phosphorylcholine by phosphatidylcholine phosphohydrolase can serve to augment the production of diglyceride by the subsequent action of phosphatidylcholine phosphohydrolase (Jenkins et al., 1994). Additionally, phosphatidic acid can be hydrolysed to diglyceride by the subsequent action of phosphatidylcholine phosphohydrolase (Morlock et al., 1991; Wu et al., 1993). It has been suggested that diglyceride generated by the sequential action of PLD and phosphatidic acid phosphohydrolase can serve to augment the production of diglyceride by phospholipase C (Exton, 1990). The formation of phosphorylcholine by the sequential action of PLD and choline kinase leads to the activation of Raf-1 kinase and mitogen-activated protein kinases (Jimenez et al., 1995). A defining characteristic of PLD is the ability to perform a transphosphatidylation reaction in the presence of primary alcohols (Heller, 1978; Kanfer, 1980). In this reaction, the phosphate group of phosphatidylcholine is transferred to the alcohol to produce phosphatidylalcohol, rather than to water to produce phosphatidic acid.

Both soluble and membrane-associated forms of PLD have been described with a wide range of molecular masses (Heller, 1978; Ella et al., 1995; Hammond et al., 1995; Wang et al., 1994). Several mechanisms have been implicated in the regulation of PLD in mammals. The analysis of regulatory mechanisms has been complicated by the possible existence of multiple isoforms of PLD, each form potentially regulated differently (see, for example, Song & Foster, 1993). Isoforms of PLD can be regulated by protein kinase C (Conricode et al., 1992; Balboa & Insel, 1995; Lopez et al., 1995) and GTP-binding proteins. Evidence supports a role in PLD regulation for ADP-ribosylation factor (Brown et al., 1993; Cockcroft et al., 1994), Rho (Ohguchi et al., 1995), Ras (Carnero et al., 1994) and Ral (Jiang et al., 1995) small GTP-binding proteins. Phosphatidylinositol 4,5-bisphosphate stimulates PLD activity (Liscovitch et al., 1994; Massenburg et al., 1994). Olate has been found to stimulate some forms of PLD (Massenburg et al., 1994) and to inhibit other forms (Waksman et al., 1996).

The gene encoding PLD in Saccharomyces cerevisiae, designated PLD1, has been cloned (Honigberg et al., 1992; Rose et al., 1993). The enzyme is a 195 kDa

Abbreviations: BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene; BDG, BODIPY-diglyceride; BPA, BODIPY-phosphatidic acid; BPBu, BODIPY-phosphatidylbutanol; BPC, 2-decanoyl-1-(O-[11-(4,4-difluoroo-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino]-indeoxy)-sn-glycerol-3-phosphocholine; BPOE, BODIPY-phosphatidylethanol; PLD, phospholipase D.
membrane-associated protein that shares limited homology with the protein from castor bean and at least one isoform of the mammalian enzyme (Hammond et al., 1995). As with the mammalian PLDs, the *Saccharomyces* enzyme (ScPLD1) is capable of performing the transphosphatidylation reaction with alcohols (Ella et al., 1995), is stimulated by phosphatidylinositol 4,5-bisphosphate (Waksman et al., 1996; Rose et al., 1995) and inhibited by oleate and chelators of divalent cations (Waksman et al., 1996). Growth on non-glucose carbon sources stimulates ScPLD1 activity (Ella et al., 1995; Waksman et al., 1996). The level of ScPLD1 activity increases rapidly when diploid *Saccharomyces* cells are suspended in sporulation medium (Ella et al., 1995). Consistent with this activation, ScPLD1 is required for sporulation: homozygous null mutants are unable to sporulate while heterozygous strains can sporulate (Ella et al., 1996; Waksman et al., 1996; Rose et al., 1995). Although loss of activity results in a decreased growth rate on non-glucose carbon sources, ScPLD1 activity is not essential for viability. Recently, a second PLD has been characterized biochemically in *S. cerevisiae* (Mayr et al., 1996; Waksman et al., 1997) and designated PLD2. PLD2 activity can be detected in a pldl mutant and is stimulated by calcium. PLD2 preferentially utilizes phosphatidylethanolamine rather than phosphatidylcholine and is unable to catalyse a transphosphorylation reaction with primary alcohols (Waksman et al., 1997). This finding suggests that in *S. cerevisiae* as with higher eukaryotes, more than one isoform of PLD may be present.

Here we report the characterization of a PLD activity in *Candida albicans*, an opportunistic human pathogen. This is the first time PLD activity has been reported in *C. albicans* an opportunistic human pathogen. The *C. albicans* enzyme possesses a membrane-associated protein that shares limited homology with the protein from castor bean and at least one isoform of the mammalian enzyme (Hammond et al., 1995). As with the mammalian PLDs, the Saccharomyces enzyme (ScPLD1) is capable of performing the transphosphatidylation reaction with alcohols (Ella et al., 1995), is stimulated by phosphatidylinositol 4,5-bisphosphate (Waksman et al., 1996; Rose et al., 1995) and inhibited by oleate and chelators of divalent cations (Waksman et al., 1996). Growth on non-glucose carbon sources stimulates ScPLD1 activity (Ella et al., 1995; Waksman et al., 1996). The level of ScPLD1 activity increases rapidly when diploid Saccharomyces cells are suspended in sporulation medium (Ella et al., 1995). Consistent with this activation, ScPLD1 is required for sporulation: homozygous null mutants are unable to sporulate while heterozygous strains can sporulate (Ella et al., 1996; Waksman et al., 1996; Rose et al., 1995). Although loss of activity results in a decreased growth rate on non-glucose carbon sources, ScPLD1 activity is not essential for viability. Recently, a second PLD has been characterized biochemically in *S. cerevisiae* (Mayr et al., 1996; Waksman et al., 1997) and designated PLD2. PLD2 activity can be detected in a pldl mutant and is stimulated by calcium. PLD2 preferentially utilizes phosphatidylethanolamine rather than phosphatidylcholine and is unable to catalyse a transphosphorylation reaction with primary alcohols (Waksman et al., 1997). This finding suggests that in *S. cerevisiae* as with higher eukaryotes, more than one isoform of PLD may be present.

Here we report the characterization of a PLD activity in *Candida albicans*, an opportunistic human pathogen. This is the first time PLD activity has been reported in *C. albicans*. A whole-cell extract was prepared from *C. albicans* strain ATCC 28367 and separated into a soluble cytosolic fraction and a particulate membrane fraction by ultracentrifugation. Each fraction was assayed in *vitro* for the presence of PLD activity using a fluorescent analogue of phosphatidylcholine, designated BPC. There was no detectable PLD activity in the cytosolic fraction while the membrane fraction produced BPA, BODIPY-phosphatidylbutanol (BPBu) and BODIPY-diglyceride (BDG) (Fig. 1). A control reaction containing commercially available peanut PLD was included to provide standards for the products. The presence of BPBu indicated that CaPLD1 was capable of performing the hallmark transphosphatidylation reaction with butanol. The transphosphatidylation reaction was examined in greater detail by assaying the ability of CaPLD1

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**METHODS**

**Strain and media.** *Candida albicans* ATCC strain 28367 was used in all experiments. Stocks were maintained on Sabouraud agar (Difco) and grown on YPD broth (Sherman, 1991). When different carbon sources were used, the base medium was YEP (Sherman, 1991) with the indicated carbon sources added to 2% (w/v).

**Preparation of extracts.** Membranes were prepared as described previously by Ella et al. (1995) with the following change: the lysis buffer contained 20 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0), 150 mM NaCl, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 5 µg leupeptin ml$^{-1}$, 1 µg pepstatin ml$^{-1}$ and 0-1 mM PMSF.

**CaPLD1 assay conditions.** *In vitro* assays using a fluorescent substrate were performed as described previously by Ella et al. (1995) with the following changes. The substrate is a BODIPY-labelled analogue of glycerophosphocholine, 2-decanoyl-1-(O-[11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino]indenyl)-sn-glycerol-3-phosphocholine (BPC) (Molecular Probes). The final reaction buffer contained 40 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0), 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0-15 mM Nonidet P-40, 1% (v/v) alcohol and 0-1 mM BPC. Each reaction contained 5 µg membrane protein as determined by a Bradford assay. The products were analysed and quantified with a FluorImager (Molecular Dynamics). The enzyme activity is presented as relative activity with respect to an appropriate control reaction. The fluorescence present in the product spots on the TLC plate was quantified with a Molecular Dynamics FluorImager and the quantity of fluorescence in the product spots from the control reaction was set to 1.0. The quantity of fluorescence in the product spots of the experimental reactions was then normalized to the value from the control reaction to obtain the relative activity. Relative activity values represent the mean of at least two independent experiments. Except where indicated, the fluorescence representing BODIPY-phosphatidic acid (BPA) and BODIPY-phosphatidylcholines (BPOR) produced in each reaction has been combined.

**Assay of CaPLD1 activity during dimorphic transition.** A culture of cells growing exponentially in YPD at 30 °C was split into three cultures. Foetal bovine serum (FBS) was added to all three to a final concentration of 25% (v/v). 1-Propanol was added to one culture to a final concentration of 0-5% (v/v) and 2-propanol was added to a second culture to a final concentration of 0-5% (v/v). The three cultures were incubated at 39 °C with agitation to induce the formation of germ tubes. Samples were taken at indicated time-points, sonicated to disrupt clumps, and examined microscopically with a haemocytometer for the appearance of germ tubes. For the assays of endogenous CaPLD1 activity during transition, a culture of exponentially growing cells at $5 \times 10^6$ cells ml$^{-1}$ in YPD at 30 °C was supplemented with either FBS to a final concentration of 25% (v/v) or N-acetylglucosamine to a final concentration of 2-5 mM and was shifted to 39 °C. Approximately $2 \times 10^7$ cells were sampled at each indicated time-point and whole-cell extracts were assayed for CaPLD1 activity as described above.

**RESULTS**

*C. albicans* possesses a membrane-associated PLD

A whole-cell extract was prepared from *C. albicans* strain ATCC 28367 and separated into a soluble cytosolic fraction and a particulate membrane fraction by ultracentrifugation. Each fraction was assayed *in vitro* for the presence of PLD activity using a fluorescent analogue of phosphatidylcholine, designated BPC. There was no detectable PLD activity in the cytosolic fraction while the membrane fraction produced BPA, BODIPY-phosphatidylbutanol (BPBu) and BODIPY-diglyceride (BDG) (Fig. 1). A control reaction containing commercially available peanut PLD was included to provide standards for the products. The presence of BPBu indicated that CaPLD1 was capable of performing the hallmark transphosphatidylation reaction with butanol. The transphosphatidylation reaction was examined in greater detail by assaying the ability of CaPLD1...
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Fig. 1. C. albicans possesses a membrane-associated PLD. Extracts of C. albicans cells were fractionated into membranes and soluble cytosol and assayed for PLD activity as described in Methods. Lanes: 1, BPC in reaction buffer without a source of PLD; 2, 5 units peanut PLD (Sigma) present; 3, Candida membranes; 4, Candida cytosol. The separation was performed on plastic-backed silica gel 60 plates (EM Science).

to utilize a variety of alcohols. As has been seen with mammalian and S. cerevisiae PLDs, CaPLD1 formed BPOR with several primary alcohols ranging from methanol to butanol (Fig. 2). CaPLD1 did not efficiently use the branched-chain alcohols 2-propanol and 2-methyl-1-propanol. In contrast, CaPLD1 was able to utilize the branched-chain alcohol 3-methyl-1-butanol very efficiently.

CaPLD1 is regulated by carbon source

ScPLD1 is regulated by growth medium with growth on non-glucose carbon sources resulting in increased activity (Ella et al., 1995; Waksman et al., 1996). To determine whether CaPLD1 was also regulated by carbon source, membranes were prepared from stationary phase cultures of C. albicans grown on rich medium with glucose, galactose, acetate or glycerol as carbon sources. Assays indicated that the carbon source affects CaPLD1. Relative to the level of activity in extracts from cells grown on glucose (1-00), CaPLD1 was stimulated by growth on galactose (1-22 ± 0-17 (± SEM) and acetate (1-41 ± 0-18) but was reduced in cells grown on glycerol (0-61 ± 0-13). The effect of additional carbon sources which C. albicans can utilize was not determined.

Fig. 2. Use of alcohols by CaPLD1. The reaction in lane 1 did not contain any extract and the reaction in lane 10 contained 5 units peanut PLD incubated with 0-5% (v/v) butanol. In the remaining lanes, extract of C. albicans cells was incubated with different alcohols, present at 0-5% (v/v). Lanes: 2, no alcohol; 3, methanol; 4, ethanol; 5, propanol; 6, butanol; 7, 2-propanol; 8, 2-methyl-1-propanol; 9, 3-methyl-1-butanol. The separation was performed on plastic-backed silica gel 60 plates (EM Science). BLPC, BODIPY-lysophosphatidylcholine.

CaPLD1 is required for dimorphic transition

The involvement of CaPLD1 in dimorphic transition was investigated using three separate approaches. The addition of exogenous PLD to culture medium has been used to artificially enhance the generation of phosphatidic acid, thus amplifying the effect of endogenous PLD activity (Kaszkin et al., 1996). Commercially purified peanut PLD was added to cultures growing in YPD at 30 and 39 °C. After 2 h, the percentage of germ tubes present in each culture was determined by direct microscopic enumeration (Fig. 3). The exogenous PLD had little effect on cells cultured at 30 °C but produced a dose-dependent stimulation of germ tube formation at 39 °C.

The presence of alcohols in the culture medium has been shown to attenuate PLD-dependent responses in mammalian cells due to the formation of phosphatidylalcohol at the expense of phosphatidic acid (Kaszkin et al., 1996). Therefore, we assessed the effect of alcohols on

Fig. 3. Exogenous PLD can stimulate dimorphic transition. Purified peanut PLD was added to cultures of C. albicans at the indicated concentrations. Following a 2 h incubation at 30 °C (●) or 39 °C (△), the percentage of cells with germ tubes in each culture was determined by direct microscopic enumeration. The results presented are from a single representative experiment.
dimorphic transition (Fig. 4). A single culture was split into three cultures and FBS was added to 25% (v/v) final concentration to induce the formation of germ tubes. One culture served as a control for the effects of alcohol and received no further additions. A second culture received 1-propanol to a final concentration of 0.5% (v/v) while the third culture received 2-propanol to 0.5% (v/v). 1-Propanol is a very good substrate for the transphosphatidylation reaction while 2-propanol is a very poor substrate. Both alcohols reduced the rate of appearance of hyphal cells (Fig. 4). Consistent with the relative ability of CaPLD1 to use the alcohols, 2-propanol, the poor substrate, had a less pronounced effect on dimorphic transition than did 1-propanol, the good substrate.

The analysis of endogenous CaPLD1 activity was investigated for additional support of a role for CaPLD1 in dimorphic transition. A culture growing at 30 °C was supplemented with FBS to 25% (v/v) and was shifted to 39 °C. Samples were withdrawn at various times after the temperature shift and membranes were prepared and assayed for CaPLD1 activity. Fig. 5(a) shows that the level of CaPLD1 activity increased following the addition of FBS and the shift in temperature. The increase in activity appeared as the germ tubes appeared in the culture. The level of increase in CaPLD1 activity was slightly less than twofold. While this induction is small, it is consistent with the level of induction seen with ScPLD1 when S. cerevisiae cells are exposed to sporulation medium (Ella et al., 1995). One possible explanation for the increase in PLD activity measured in the assay is the presence of a PLD in the FBS. Incubation of BPC with serum failed to produce any detectable BPA (data not shown).

FBS could alter CaPLD1 activity through a number of different responses, not just the induction of dimorphic transition. To clarify the correlation between CaPLD1 activity and dimorphic transition, the previous experiment was repeated with a more defined inducer. Culturing C. albicans cells at 39 °C in the presence of 2.5 mM N-acetylglucosamine is sufficient to induce the appearance of germ tubes (Paveto et al., 1990). CaPLD1 activity was observed to increase and then decrease with similar kinetics when transition was induced with temperature and N-acetylglucosamine (Fig. 5b).

The change in CaPLD1 activity seen in these experiments could indicate changes associated with adaptation to growth at elevated temperature. To determine whether this was the case, the level of CaPLD1 activity in cells cultured at 39 °C for an extended time was compared to a parallel culture maintained at 30 °C. The level of
CaPLD1 activity was actually 23% lower in cells cultured overnight at 39 °C relative to cells cultured at 30 °C. Therefore, the observed stimulation in CaPLD1 activity correlated with the induction of germ tubes and not with growth at elevated temperatures.

**DISCUSSION**

This is the first report of PLD activity in the opportunist fungal pathogen *C. albicans*. In light of the number of organisms possessing more than one form of PLD, we have designated the enzyme described in this report CaPLD1. This enzyme is membrane-associated and capable of performing a transphosphatidylidation reaction in the presence of primary alcohols. CaPLD1 is stimulated by growth on galactose or acetate but inhibited by growth on glycerol. CaPLD1 activity is stimulated during dimorphic transition and induced by elevated temperature and either serum or N-acetylglucosamine. Previous reports on phospholipase activity in *C. albicans* (for example, Barrett-Bee *et al.*, 1985; Ibrahim *et al.*, 1995) have focused on secreted enzymes that may facilitate tissue invasion, which may profoundly augment virulence (Ibrahim *et al.*, 1995). None of these earlier reports have described PLD activity associated with *C. albicans*. CaPLD1 activity described in this report appears to be required for the expression of another virulence factor, the completion of transition from yeast to hyphal cell morphology. This conclusion is based on the ability of exogenous PLD to stimulate the process, the ability of a primary alcohol in the medium to attenuate the process, and the stimulation of CaPLD1 activity by inducers of the process. A direct effect of the alcohol on dimorphic transition cannot be excluded. For example, the alcohols could modify the cell membrane by interacting with the membrane lipids, thus altering the cell’s ability to change shape.

*C. albicans* and *S. cerevisiae* are very similar in many ways. Both are budding yeasts capable of undergoing dimorphic transitions to elongated growth patterns which result in the formation of germ tubes or pseudo-hyphae, respectively (Soll, 1986; Gimeno *et al.*, 1992). The similarity is such that many *C. albicans* genes have been cloned by complementation of the homologous mutants in *S. cerevisiae* (Kurtz *et al.*, 1988). Therefore, it is not surprising that CaPLD1 shares many features with ScPLD1. Both enzymes are membrane-associated, capable of transphosphatidylidation with alcohols, and regulated by carbon source. The major difference between these two enzymes is their physiological role, and even in this regard there is similarity.

Diploid *S. cerevisiae* cells experiencing nutrient deprivation, particularly lack of nitrogen and glucose, will undergo meiosis and sporulation to form haploid spores. ScPLD1 is stimulated in response to conditions that induce this differentiation and is essential for the completion of the process (Ella *et al.*, 1995; Waksman *et al.*, 1996; Rose *et al.*, 1995). *C. albicans* does not undergo meiosis and sporulation to form haploid spores; consequently, CaPLD1 must play a different role. In a similar manner, CaPLD1 activity is stimulated by inducers of dimorphic transition and is essential for the completion of the change. In a possibly similar manner, a phosphatidylinositol/phosphatidylcholine transfer protein is required for dimorphic transition in the yeast *Yarrowia lipolytica* (Lopez *et al.*, 1994). Like PLDs, phosphatidylinositol/phosphatidylcholine transfer proteins can alter the phospholipid composition of secretory vesicles to control physiological processes. These findings indicate the importance of lipid composition and signalling in the control of differentiation in fungi. The involvement of CaPLD1 in dimorphic transition and of ScPLD1 in sporulation suggests that one role for fungal PLDs may be the conversion of external stimuli into developmental changes. A role for PLD activity in differentiation is not limited to fungi. PLD plays important roles in the differentiation of rat preovulatory follicles (Liscovitch & Amsterdam, 1989; Amsterdam *et al.*, 1994) and murine haematopoietic cells (Clejan *et al.*, 1996). The relative simplicity of the fungal systems will facilitate the dissection of the role of PLD in eukaryotic cell differentiation.

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