Phosphatidylinositol-4-phosphate 5-kinase activity is stimulated during temperature-induced morphogenesis in *Candida albicans*

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Phosphoinositides are important lipid signalling molecules in eukaryotic cells. Phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) catalyses the production of phosphatidylinositol 4,5-bisphosphate (PIP₂), which stimulates phospholipase D1 (PLD1) activity in mammalian and yeast cells. PLD1 catalyses the formation of phosphatidic acid (PA), which has been shown to activate PI4P5Ks in mammalian and *Saccharomyces cerevisiae* cells. In the present study, PI4P5K activity in the opportunistic pathogen *Candida albicans* was identified. A gene with significant sequence homology to the *S. cerevisiae* PI4P5K was cloned and designated *MSS4*. This gene was demonstrated to encode a functional PI4P5K by expression in *S. cerevisiae*. This enzyme was found to be membrane-associated and was stimulated by PA. Within the first 20 min after induction of polarized hyphal growth induced by a shift to elevated temperature, PI4P5K activity increased 2.5-fold. This stimulation was not observed when hyphae were induced by a combination of elevated temperature and serum. A lack of PLD1 activity resulted in the loss of induction of PI4P5K activity during the morphogenetic switch. Furthermore, the addition of propranolol attenuated the stimulation of PI4P5K activity during morphogenesis. These results suggest that PA derived from PLD1 activity stimulates *C. albicans* PI4P5K during the switch to the hyphal form under some conditions.

Keywords: dimorphic transition, phospholipase D, PLD1, *MSS4*

INTRODUCTION

Phosphoinositides are found in all eukaryotic cells and are crucial molecules in many cellular processes (Majerus, 1992). These molecules represent only a small portion of the total membrane lipid content. Nevertheless they have emerged as key players in cytoskeletal organization, cell proliferation, vesicular trafficking and lipid signal transduction pathways (reviewed by Fruman *et al.*, 1998; Odorizzi *et al.*, 2000). A vital component in phosphoinositide signalling is phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₂ has a fundamental role in many cellular processes. One of the most significant roles for PIP₂ is as a source for the important second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate following hydrolysis by phospholipase C

(PLC). DAG is known to activate several isoforms of protein kinase C in mammalian systems and is important in cellular secretion (Liscovitch *et al.*, 1999; Siddhanta & Shields, 1998), while inositol 1,4,5-trisphosphate releases intracellular calcium stores. In addition, PIP₂ regulates the actin cytoskeleton by binding and regulating various actin-binding proteins (Janmey, 1994) such as profilin (DiNubile & Huang, 1997a, b), gelsolin (Janmey & Stossel, 1987) and α -actinin (Fukami *et al.*, 1992) in vertebrates. The modulation of the activity of other proteins is another function of PIP₂: phospholipase D1 (PLD1) activity is stimulated by PIP₂ in mammalian (Liscovitch *et al.*, 1994), *Saccharomyces cerevisiae* (Rose *et al.*, 1995; Waksman *et al.*, 1996) and *Candida albicans* cells (McLain & Dolan, 1997). Roles for proteins that contain a pleckstrin homology domain have also been described in PIP₂ signalling. It has been demonstrated, for example, that the PLC δ 1 pleckstrin homology domain has a high affinity for PIP₂ (Isakoff *et al.*, 1998) and that the pleckstrin homology domain is necessary and sufficient for PLC δ 1 attachment to membranes

Abbreviations: DAG, 1,2-diacylglycerol; LPP, lipid phosphate phosphohydrolases; PA, phosphatidic acid; PI4P5K, phosphatidylinositol-4-phosphate 5-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD1, phospholipase D1.

(Varnai & Balla, 1998). Due to the wide range of roles played by PIP₂, its synthesis and degradation must be tightly regulated by the cell.

PIP₂ is synthesized by the sequential phosphorylation of phosphatidylinositol by phosphatidylinositol-4-kinase and phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) at the D-4 and D-5 position of the inositol ring, respectively. PIP₂ can also be synthesized by phosphatidylinositol-5-phosphate 4-kinase (from phosphatidylinositol 5-phosphate) (Rameh *et al.*, 1997). Originally, type I and type II PI4P5Ks were described in mammals (Loijens & Anderson, 1996); however, the substrate specificity of these enzymes has revealed that the type II enzymes are phosphatidylinositol-5-phosphate 4-kinases (Rameh *et al.*, 1997). In mammals, different isoforms of PI4P5K have been identified and designated α , β and γ (Ishihara *et al.*, 1996, 1998; Loijens & Anderson, 1996; Loijens *et al.*, 1996). Acidic phospholipids such as phosphatidic acid (PA) (Ishihara *et al.*, 1996, 1998; Jenkins *et al.*, 1994; Moritz *et al.*, 1992) and phosphatidylserine (Cochet & Chambaz, 1986) have been shown to stimulate PI4P5K in mammalian cells. Additional regulators of PIP₂ synthesis include the small GTP-binding proteins Rho and Rac (Chong *et al.*, 1994; Tolias *et al.*, 2000). Honda *et al.* (1999) demonstrated recently that PI4P5K α is a downstream effector of ADP-ribosylation factor ARF6 and is involved in membrane ruffling.

The *S. cerevisiae* PI4P5K is encoded by the gene *MSS4* and is a 779 aa protein (Desrivieres *et al.*, 1998; Homma *et al.*, 1998). The yeast PI4P5K is required for actin cytoskeleton assembly as mutants fail to form normal actin filaments and fail to localize the cytoskeleton properly during polarized cell growth (Desrivieres *et al.*, 1998; Dove *et al.*, 1997; Homma *et al.*, 1998). Loss of PI4P5K is suppressed by overexpression of *RHO2*, a Rho-type GTPase involved in the regulation of the actin cytoskeleton, implicating Rho2p as a downstream effector of PI4P5K (Desrivieres *et al.*, 1998). As in mammalian systems, PI4P5K in *S. cerevisiae* has been reported to be stimulated by PA (Homma *et al.*, 1998). PA is a putative second messenger that has been implicated in the regulation of lipid metabolism (Liscovitch *et al.*, 1999), cell proliferation (Kaszkina *et al.*, 1996), differentiation (Amsterdam *et al.*, 1994), secretion (Siddhanta & Shields, 1998) and actin polymerization (Ha & Exton, 1993). PA can be produced by the hydrolysis of the major membrane phospholipid, phosphatidylcholine, by signal-activated PLD1. Since PI4P5K catalyses the production of PIP₂, which is required for the maximal activity of PLD1, and PLD1 catalyses the production of PA, which is required for the maximal activity of PI4P5K, the sequential action of PLD1 and PI4P5K could result in a feedback loop that would stimulate the production of both PA and PIP₂ (Liscovitch *et al.*, 1994).

C. albicans is the most frequently isolated fungal pathogen in humans. Infections caused by this organism are a major cause of morbidity and mortality among immunocompromised patients, women of childbearing age, infants and the elderly. Antifungal antibiotics are available; however, many have the potential to cause

liver and/or kidney toxicity in humans. Strains of *C. albicans* that are resistant to the most commonly used antifungals are appearing with increasing frequency (Rex *et al.*, 1995). It is therefore essential that the mechanisms involved in *C. albicans* virulence be elucidated. This organism can grow as budding yeast-form cells or as elongated hyphae, depending on growth conditions. Virulence has been associated with the ability to undergo conversion from the round yeast-form to the elongated hyphal form in a morphogenetic process termed dimorphic transition (Cutler, 1991; Navarro-Garcia *et al.*, 2001). High temperature, neutral pH and serum are potent inducers of dimorphic transition (Ernst, 2000; Odds, 1988). The initial stage of dimorphic transition is the formation of a germ tube, which is an elongated daughter cell formed by an existing yeast-form cell. It has been proposed that hyphae have a mechanical advantage in the penetration of host-cell membranes and the destruction of phagocytes (Odds, 1988). Consequently, molecules found to regulate the process of dimorphic transition may be effective targets for improved antifungal drugs (Leberer *et al.*, 1997). In this report, PI4P5K activity in *C. albicans* is described. This enzyme is stimulated by PA and during temperature-induced morphogenesis. Finally, we present evidence that stimulation during morphogenesis requires PLD1 activity.

METHODS

Strains, media and preparation of extracts. *C. albicans* strains used were CAF2-1 (*ura3::imm434/URA3*) and JDC12 (strain also designated *pld1 Δ 1* in Hube *et al.*, 2001; *ura3::imm434/ura3::imm434 pld1::hisG::URA3::hisG/pld1::hisG*), which are isogenic except as indicated (Hube *et al.*, 2001). *S. cerevisiae* strains used were 23567 (*a/ α his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 1 met15 Δ 0/+ lys2 Δ 0/+ ura3 Δ 0/ura3 Δ 0 mss4::Kan^R/+; Research Genetics), SEY6210.1 (*MATa leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ)* and AAY202.1 (SEY6210.1 with *mss4 Δ ::HIS3MX6* carrying YEplac-*mss4-102*). Stocks were maintained on YPD agar (Sherman, 1991; Difco), grown in YPD broth at 30 °C; 26 °C was used for the *mss4-102* temperature-sensitive mutants. *S. cerevisiae* strains were transformed with plasmid pCW20 and empty vector (YEplac352) using a lithium acetate protocol (Gietz & Woods, 2001). Whole-cell extracts were prepared as described previously (McLain & Dolan, 1997). For localization experiments, CAF2-1 whole-cell extract was fractionated by centrifugation at 10000 g for 30 min. The resulting soluble and pellet fractions were assayed for PI4P5K activity. For determination of detergent solubility, Triton X-100 was added to 1% (w/v) to the whole-cell extract prior to centrifugation.*

Morphogenesis assay. To measure the effect of the yeast-to-hyphae transition on enzyme activity, PI4P5K and PLD1 *in vitro* assays were performed with extracts from wild-type cells induced to form hyphae by temperature shift from 30 to 37 °C. Overnight cultures of CAF2-1 and JDC12 in YPD at 30 °C were diluted 1:100 in fresh YPD medium and incubated at 37 °C for 90 min. Samples were taken at increasing times post-induction and whole-cell extracts were prepared and assayed for both PI4P5K and PLD1 activities. Where indicated, morphogenesis was induced by temperature shift and the addition of fetal bovine serum to a final concentration of 5% (v/v).

PI4P5K assay. PI4P5K activity was assayed according to published methods used for the *S. cerevisiae* enzyme (Homma *et al.*, 1998). Briefly, *C. albicans* whole-cell extracts containing 55 µg protein, as determined by the Bradford assay, were incubated with 80 µM PI4P (Sigma) and 5 µCi [γ - 32 P]ATP (NEN) for 10 min at 30 °C. Reactions were incubated with or without 50 µM PA where explicitly stated. The reactions were terminated and the lipids extracted by the addition of chloroform/methanol according to the method of Bligh & Dyer (1959). The lipid products were separated on a silica gel thin layer chromatography plate impregnated with 1.2% potassium oxalate (v/v) and developed with chloroform/methanol/sodium hydroxide/water (40:42:5:10, by vol.) (Homma *et al.*, 1998). Quantification of radioactivity incorporated into products was performed with a Molecular Dynamics PhosphorImager. A second assay for PI4P5K used the substrate NBD-PI4P (Echelon Laboratories), which is a fluorescent analogue of the *in vivo* substrate for PI4P5K. Micelles containing the substrate were prepared as described for the fluorescent PLD1 substrate by McLain & Dolan (1997) and the reactions were performed as with the radioactive assay. Control samples were always run on the same TLC plate as the experimental samples and both control and experimental samples were scanned at the same time. Values were normalized to controls on the same TLC plate and these normalized values were used to compare results between plates. Statistical analysis was performed using the Student's *t*-test for three independent experiments with duplicate samples in each.

Fluorescent PLD1 assay. PLD1 activity was assayed using whole-cell extracts and the fluorescent substrate BODIPY-phosphatidylcholine as described previously (McLain & Dolan, 1997).

DNA sequence analysis. Homology searches were conducted with the BLAST algorithm. Sequence alignments were performed with DNASTAR MEGALIGN software version 1.05 (DNASTAR) using the CLUSTAL W 1.6 method. A rooted phylogenetic tree was generated using the CLUSTAL W method with the PAM250 residue weight table.

Construction of plasmids. The DNA encoding the putative *C. albicans* Mss4p was amplified from *C. albicans* SC5314 genomic DNA by PCR. The primers amplifying the upstream untranslated and 5' coding region of MSS4 included restriction sites *Eco*RI and *Xba*I at the 5' ends for cloning. Primer sequences were 5'-GCATATGAATTCCATCGCTCTCCA-TCAACACC-3' and 5'-GAACATCTAGAACTGCCACT-C-3' which contained the restriction sites *Eco*RI and *Xba*I (bold type), respectively. The digested PCR product was cloned into the *Eco*RI-*Xba*I sites of the *S. cerevisiae*/*E. coli* shuttle vector YEp352, generating pCW10. The 3' coding region of MSS4 and its terminator were amplified using primers 5'-GCAGTTTCTAGATGTTCTGGAG-3' and 5'-CATGGCAAGCTTTCGGTTGGTCTGAATCAC-3' which contained the restriction sites *Xba*I and *Hind*III (bold type), respectively. The digested PCR product was inserted into the *Xba*I/*Hind*III sites of pCW10 to generate plasmid pCW20.

RESULTS

C. albicans PI4P5K is regulated by PA

PI4P5K activity is readily detected in *S. cerevisiae* using a radioactive kinase assay described by Homma *et al.* (1998). To determine the level of PI4P5K activity in *C. albicans*, cells were assayed for this kinase as described previously (Homma *et al.*, 1998). PI4P5K activity is

Fig. 1. *C. albicans* PI4P5K is stimulated by PA and by Triton X-100. (a) Autoradiograph of a representative TLC plate showing the effects of PA on PI4P5K activity. (b) The effect of Triton X-100 on PI4P5K activity. PI4P5K activity is presented in arbitrary units (AU) based on the intensity of fluorescence of the NBD-PIP₂ spots.

stimulated by PA in other organisms (Desrivieres *et al.*, 1998; Homma *et al.*, 1998; Ishihara *et al.*, 1996, 1998; Jenkins *et al.*, 1994; Moritz *et al.*, 1992); therefore, PA was added to reactions to ascertain whether the PI4P5K in *C. albicans* was stimulated by acidic phospholipids. The results indicated a 48% increase in the formation of radioactive PIP₂ in extracts incubated with exogenous PA as compared to control extracts ($n=6$; $P=0.036$) (Fig. 1a). Thus, as with the mammalian and *S. cerevisiae* enzymes, *C. albicans* expresses a PI4P5K that requires PA for maximal activity.

PI4P5K is membrane-associated

To examine the intracellular localization of PI4P5K, we first analysed the effect of Triton X-100 on PI4P5K activity (Fig. 1b). Similar to published results for the *Schizosaccharomyces pombe* enzyme (Vancurova *et al.*, 1999), our results show a stimulation by Triton X-100 at concentrations above 1% (v/v). Cell fractionation experiments were subsequently performed. In the absence of detergent, the pellet (particulate) fraction

Fig. 2. Both PI4P5K and PLD1 activity increase in cultures undergoing morphogenesis. (a) Wild-type *C. albicans* cultures were induced to undergo the switch to polarized hyphal growth. Data shown are from a single representative experiment because the absolute timing of the stimulation varied slightly between experiments. The overall pattern and magnitude of the changes in activity were highly reproducible ($n=6$). PI4P5K activity (■) is presented in arbitrary units (AU) based on the intensity of fluorescence of the NBD-PIP₂ spots. PLD1 activity (□) is stimulated during morphogenesis. Whole-cell extracts of cultures induced to form germ tubes by a temperature shift from 30 to 37 °C were assayed for PLD1 activity at various time points post-induction. PLD1 activity is presented in arbitrary units (AU) based on the intensity of fluorescence of the BODIPY-PA spots. The percentage of cells with germ tubes is indicated at each time point (○). (b) PI4P5K activity was measured in cells that had been induced by both temperature and serum. Samples were taken at time points after induction and the formation of germ tubes (○) and PI4P5K activity (■) were determined. Data are from a representative experiment.

contained 67.7% of the total PI4P5K activity, whereas the soluble fraction contained 32.3% of the total activity. When Triton X-100 was added to solubilize membrane proteins prior to fractionation, PI4P5K activity in the particulate fraction decreased to 27%,

Fig. 3. Propranolol inhibits PI4P5K activation during dimorphic transition. Wild-type *C. albicans* cultures were induced to form germ tubes by a temperature shift from 30 to 37 °C. Propranolol (2 mM) was added to cultures at the temperature shift. (a) PI4P5K activity (■) was attenuated with the addition of propranolol in comparison with control cells from Fig. 2. Results presented are from a single representative experiment. (○), Germ-tube formation. (b) The doubling time was established to verify that propranolol addition did not inhibit germ-tube formation by increasing the generation time. A slight increase in growth rate was noted with 2 mM propranolol which was statistically insignificant. Data are from three independent sets of extracts with each extract assayed twice (total of six assays for each condition). Error bars indicate the standard error of the mean.

which coincided with an increase in activity in the Triton X-100-soluble fraction to 73% of the total activity. The presence of PI4P5K in the Triton X-100-solubilized fraction indicates that the enzyme is associated with membranes. Although the specific membrane to which PI4P5K is bound has not been identified, these data are consistent with the plasma membrane localization reported for the *S. cerevisiae* (Homma *et al.*, 1998), *Schizosaccharomyces pombe* (Vancurova *et al.*, 1999) and mammalian enzymes (Honda *et al.*, 1999).

Fig. 4. PI4P5K is not induced in PLD1-deficient cell cultures undergoing morphogenesis. *pld1Δ* mutant cultures were induced to undergo dimorphic transition by temperature shift. ■, PI4P5K activity; ○, percentage of cells with germ tubes. Data shown are from a single representative experiment because the absolute timing of the stimulation varied slightly between experiments. The overall pattern and magnitude of the changes in activity were reproducible. PI4P5K activity is presented in arbitrary units based on the intensity of fluorescence of the NBD-PIP₂ spots.

PI4P5K activity is stimulated during morphogenesis

Earlier experiments performed in this laboratory have shown that PLD1 plays a significant role in the morphogenetic process (Hube *et al.*, 2001; McLain & Dolan, 1997). Given that the product of PLD1 catalysis is PA

and that PA stimulates PI4P5K, we aimed to assess a possible correlation between PI4P5K and PLD1 during the morphogenetic switch. *C. albicans* whole-cell extracts were assayed during morphogenesis to determine the level of PI4P5K activity at various time points following induction. These experiments were performed with a fluorescent assay for PI4P5K activity using NBD-PI4P as the substrate for the reaction. The results indicated a maximum level of PI4P5K activity at the 20 min time point, yielding a 2.5-fold increase in activity (Fig. 2a); this increase over the zero time point was statistically significant ($P < 0.01$; $n = 6$). Maximum PI4P5K activity was coincident with the initiation of germ tube emergence. At 90 min post-induction there was another increase in PI4P5K activity when ~90 % of cells exhibited germ tubes. In a parallel experiment, cells were inoculated into fresh medium and incubated at 30 °C to detect changes in PI4P5K due to a return to growth. No significant changes were detected in PI4P5K activity under these conditions (data not shown). The small magnitude of stimulation raises the question of physiological impact. By way of comparison, PLD1 activity is stimulated only 60 % during morphogenesis, but the null mutant is unable to form hyphae under specific conditions and is avirulent in a mouse model system (Hube *et al.*, 2001; McLain & Dolan, 1997). Thus, there is a solid precedent, supported by both biochemical and genetic analysis, for small changes in activity having relevant and significant physiological impact in the process of morphogenesis in *C. albicans*.

To determine whether the increase in PI4P5K activity was a non-specific effect of growth at elevated temperatures, cells were induced to form germ tubes by the combination of elevated temperature and serum. The

Table 1. Percentage amino acid similarity of full-length members of the PI4P5K family

Abbreviations and accession numbers are as follows: *Arabidopsis thaliana* (At), T51821; *C. albicans* (Ca), CaMSS4; *Caenorhabditis elegans* (Ce), CAA91791.1; *Drosophila melanogaster* (Dm), AAC25576.1; *Homo sapiens* alpha (Hsα), XP_001338.2; *Homo sapiens* beta (Hsβ), XP_015261.1; *Mus musculus* alpha (Mmα), NP_032872.1; *Mus musculus* beta (Mmβ), NP_032873.1; *Mus musculus* gamma (Mmγ), NP_032870.1; *Nicotiana rustica* (Nr), AAF80332.1; *Oryza sativa* (Os), AAG60194.1; *Saccharomyces cerevisiae* (Sc), P38994; *Schizosaccharomyces pombe* (Sp), CAB10125.1; *Sus scrofa domestica* (Ssd), AAB53645.1.

	At	Ca	Ce	Dm	Hsα	Hsβ	Mmα	Mmβ	Mmγ	Nr	Os	Sc	Sp	Ssd
At	–	25.8	11.1	18.9	22.2	19.2	21.5	20.9	17.2	74.8	14.3	17.3	20.9	20.4
Ca		–	12.3	23.9	22.3	21.5	21.9	22.1	21.1	27.8	10.1	48.5	45.2	24.1
Ce			–	11.3	10.9	11.0	11.7	11.2	12.1	10.4	10.5	10.7	11.7	12.3
Dm				–	49.2	41.5	44.2	46.3	38.7	19.6	10.5	15.1	18.7	19.7
Hsα					–	59.9	60.1	91.4	65.8	23.5	10.5	22.0	24.3	24.1
Hsβ						–	95.9	56.2	57.6	18.8	10.3	17.3	18.3	23.4
Mmα							–	56.6	60.3	19.9	9.4	18.6	22.1	23.2
Mmβ								–	63.4	22.0	10.3	19.8	23.6	23.4
Mmγ									–	17.7	12.4	14.5	19.2	23.6
Nr										–	17.3	18.5	20.5	22.4
Os											–	11.1	11.1	11.3
Sc												–	31.0	24.6
Sp													–	22.9
Ssd														–

Fig. 5. *C. albicans* PI4P5K protein is homologous to PI4P5K family members. (a) Alignment of the kinase homology domains of selected PI4P5K family members. Highly conserved residues are shown on a black background. Amino acid residue numbers are annotated on the left. GXSGS, a putative nucleotide-binding motif; MDYSL, a moiety important for catalysis; and IID, a putative Mg^{2+} - and Mn^{2+} -binding motif are indicated above each consensus sequence. (b) Phylogenetic tree for PI4P5Ks of various species. Horizontal distances correspond to genetic distance. See Table 1 for the percentage amino acid similarity of full-length members of the PI4P5K family.

results of a representative experiment are shown in Fig. 2(b). As expected, the kinetics and extent of germ-tube formation was nearly identical to that seen with temperature alone (Fig. 2a). PI4P5K activity responded to the complex stimuli in a qualitatively similar manner: activity increased during the first 20 min, declined and rose again at 90 min post-induction. However, unlike the results with temperature stimulation alone, the quantitative changes induced by temperature and serum lacked statistical significance.

Propranolol inhibits PI4P5K induction during morphogenesis

To determine whether PA was activating PI4P5K *in vivo*, propranolol was added to cultures coincident with the induction of morphogenesis. Propranolol has been used at low concentrations (0.1–0.2 mM) to inhibit mammalian lipid phosphate phosphohydrolases (LPPs) that convert PA into DAG. The LPPs in *C. albicans* are

not affected by low concentrations of propranolol (*C. A. Baker, K. Desrosiers & J. W. Dolan, unpublished*); consequently, higher concentrations (1–5 mM) were used. At the higher concentrations, propranolol can act by binding to the PA and rendering it inaccessible to downstream targets of PA (*C. A. Baker, K. Desrosiers & J. W. Dolan, unpublished; Surewicz & Leyko, 1981*). As expected, propranolol attenuated PI4P5K stimulation throughout the dimorphic transition (Fig. 3a) relative to untreated controls (Fig. 2a). The addition of 2 mM propranolol also eliminated germ-tube formation in these cultures (Fig. 3a). To rule out the possibility that the inhibition of germ-tube formation was the result of reduced proliferation, growth rates were determined at increasing propranolol concentrations. The addition of propranolol in concentrations up to 1 mM did not affect growth rates. However, 2 mM caused a slight increase in generation time (Fig. 3b). Thus, propranolol reduced PI4P5K activation and germ-tube formation without substantially affecting the growth rate.

Correlation of PI4P5K and PLD1 activity

In light of the fact that both PLD1 and PI4P5K are activated during morphogenesis (McLain & Dolan, 1997), PLD1 activity was assayed in the same extracts used to determine PI4P5K activation (Fig. 2a). The timing of activation revealed that PI4P5K activity is stimulated earlier than PLD1 and to a larger degree, suggesting that PIP₂ generated by PI4P5K could account for the subsequent stimulation of PLD1.

To determine whether PLD1 was playing a role in the stimulation of PI4P5K, a homozygous *pld1Δ* knockout strain, JDC12 (Hube *et al.*, 2001), was assayed for PI4P5K activity during morphogenesis. PI4P5K activity was observed to decline steadily during morphogenesis in the *pld1Δ* cells (Fig. 4). Although PA can be generated by the phosphorylation of DAG by diglyceride kinase (Morlock *et al.*, 1991; Wu *et al.*, 1993) and by acylation of monoacyl-glycerol 3-phosphate (Athenstaedt *et al.*, 1999), this complete lack of induction of PI4P5K activity in *pld1Δ* cells indicates that PLD1 supplies PA necessary for PI4P5K stimulation during dimorphic transition to elongated hyphae.

C. albicans carries a gene that is highly homologous to PI4P5K family members

An ORF on contig 6-1966 was found by a BLAST search of the *C. albicans* sequencing project (<http://www-sequence.stanford.edu/group/candida>) that was predicted to encode a protein closely related to Mss4p of *S. cerevisiae*. The ORF is 3326 bp in length, encodes 491 aa and is 54 % identical and 69 % similar to the *S. cerevisiae* protein over a region of 508 aa of the *S. cerevisiae* protein (Table 1). A region of 347 aa contains several motifs typical of kinases. Of the 347 aligned residues, 31 are conserved in all PI4P5K homologues examined; an alignment of this region from three fungal homologues and one human homologue is shown in Fig. 5(a). These conserved residues are likely to be involved in phosphotransferase activity or are strategically located to interact with the substrate, PI4P, in the active site. *C. albicans* PI4P5K has a glycine-rich loop with the consensus sequence of GXSGS (residues 195–199) and a conserved lysine (residue 212). These residues resemble the phosphate-binding loop of protein kinases and other nucleotide-binding proteins (Anderson *et al.*, 1999). As with other kinases, *C. albicans* and other PI4P5K family members share an aspartic acid (D) residue (347 in *C. albicans*) within the consensus sequence MDYSL. This aspartic acid residue corresponds to D166, which is important for catalysis in protein kinase A (Knighton *et al.*, 1991). Another aspartic acid residue (436) is present within the consensus sequence IID in the *C. albicans* protein and other homologues and coincides with D184 in protein kinase A that binds Mg²⁺ and Mn²⁺ ions (Knighton *et al.*, 1991).

A phylogenetic tree was generated based on the homology of kinase domains of known or suspected PI4P5Ks to better understand the relationships within this gene

Fig. 6. The *C. albicans* homologue of *MSS4* suppresses the temperature-sensitivity of an *S. cerevisiae* *mss4^{ts}* mutant. Relevant phenotypes of tested strains are shown in Table 2. (a) Key to strains tested for growth at permissive (26 °C) and non-permissive (38 °C) temperatures. The 'ts + CaMSS4' strains represent three isolates carrying the plasmid pCW20. (b) Strains were tested for growth on the indicated media. Photographs were taken after 2 days of incubation.

family (Fig. 5b). The tree contained three prominent clusters. The first cluster was composed of fungal homologues, including *C. albicans* Mss4p, as well as the porcine kinase. The second cluster contained human, mouse and *Drosophila melanogaster* PI4P5Ks. The third cluster contained plant homologues.

C. albicans *MSS4* can complement a *S. cerevisiae* *mss4^{ts}*

To determine whether the cloned ORF encodes a functional homologue of the *S. cerevisiae* PI4P5K Mss4p, we constructed a plasmid (pCW20) carrying this gene

Table 2. Relevant phenotypes of tested strains

Strain	Leu	Ura	Mss4
Wild-type (wt)	—	—	+
<i>mss4</i> Δ YEplac- <i>mss4</i> -102	+	—	ts
<i>mss4</i> Δ YEplac- <i>mss4</i> -102 YEpl352	+	+	ts
<i>mss4</i> Δ YEplac- <i>mss4</i> -102 pCW20	+	+	CaMSS4

under the transcriptional control of its own putative promoter. The amino acid sequence has a serine residue in the C terminus at position 463 that is encoded by non-standard CUG codon usage in *C. albicans* (Ohama *et al.*, 1993; Santos *et al.*, 1993). pCW20 was transformed into *S. cerevisiae* strains SEY6210.1 (MSS4) and AAY202.1 (*mss4*Δ::HIS3 carrying YEplac-*mss4*-102^{ts}) and the transformants were tested for the ability to suppress the temperature sensitivity of the *mss4*-102 allele (Fig. 6 and Table 2). The temperature-sensitive transformants carrying pCW20, which contains the *URA3* gene as a selectable marker, were unable to grow on plates containing 5-fluoroorotic acid, indicating that the *Candida* MSS4 gene was required for viability at the non-permissive temperature (38 °C). Since these strains carried the *mss4*-102 temperature-sensitive allele, the cells should be able to lose pCW20 and grow on 5-fluoroorotic acid at the permissive temperature of 26 °C. The pCW20 transformants were able to grow on 5-fluoroorotic acid at 26 °C, forming colonies after extended incubation (data not shown). The slow growth suggests that even at the permissive temperature, the *mss4*-102 cells benefit from the added activity provided by the *C. albicans* MSS4 gene on pCW20. Plasmid pCW20 was also used to transform *S. cerevisiae* strain 23567, a diploid that is heterozygous for MSS4 (*MSS4*/*mss4*::Kan^R). These transformants showed an approximately 40% increase in PI4P5K activity that returned to untransformed levels following loss of pCW20. Taken together, these results confirm that the *C. albicans* gene designated MSS4 encodes a functional PI4P5K. The presence of additional *C. albicans* genes encoding PI4P5K activity cannot be excluded, but no other regions with significant homology to PI4P5Ks have been identified in the genome sequence.

DISCUSSION

In the present study, PI4P5K activity in the pathogenic fungus, *C. albicans* was identified. As in *S. cerevisiae* (Desrivieres *et al.*, 1998; Homma *et al.*, 1998), murine and bovine cells (Ishihara *et al.*, 1996, 1998; Jenkins *et al.*, 1994; Moritz *et al.*, 1992), *C. albicans* PI4P5K is stimulated by PA. In addition, we have found this PI4P5K, like other known PI4P5Ks (Anderson *et al.*, 1999; Homma *et al.*, 1998; Honda *et al.*, 1999; Vancurova *et al.*, 1999), is membrane-associated (Fig. 1). A gene, designated MSS4, has been cloned and demonstrated to encode a PI4P5K activity when expressed in *S. cerevisiae*.

Our results suggest that PI4P5K might play a role in *C. albicans* transition to polarized growth in response to some, but not all, inducing stimuli. The exact role PI4P5K would play in this process is unknown; however, the change of shape from an ellipsoid to an elongated germ tube involves actin cytoskeleton reorganization. The product of PI4P5K activity, PIP₂, could affect cell polarization in a number of ways. For example, PIP₂ has been identified as a lipid that dissociates capping proteins from the barbed ends of actin filaments, thus promoting actin assembly (Heiss & Cooper, 1991; Janmey *et al.*, 1987; Janmey & Stossel, 1987). Profilin is an actin-binding protein that binds PIP₂. It is required for the maintenance of cell polarity and is essential for the proper formation of actin cables that occur at areas of active cellular growth (DiNubile & Huang, 1997a, b). Thus, PI4P5Ks in yeast and murine models have been shown to be critical for actin assembly (Audhya *et al.*, 2000; Desrivieres *et al.*, 1998; Homma *et al.*, 1998; Tolias *et al.*, 2000). It is therefore plausible that *C. albicans* PI4P5K is stimulated to provide increased levels of PIP₂ for the actin remodelling process.

PI4P5K stimulation during morphogenesis is attenuated by the addition of propranolol (Fig. 3). Propranolol is typically used in mammalian cells at low concentrations (100–200 μM) to inhibit LPP, the enzyme that hydrolyses PA to DAG (Meier *et al.*, 1998; Morlock *et al.*, 1991; Wu *et al.*, 1993). Frequently, inhibition by propranolol suggests that DAG, rather than PA, is the essential molecule in a cellular process. In contrast to mammalian LPP, yeast LPP is relatively insensitive to propranolol (C. A. Baker, K. Desrosiers & J. W. Dolan, unpublished; Ella *et al.*, 1995). An earlier study using fluorescent spectroscopy and millimolar concentrations of propranolol revealed that the drug bound directly to several phospholipids, particularly PA and phosphatidylserine (Surewicz & Leyko, 1981). In addition, fluorimetry studies have shown that the intrinsic fluorescence of propranolol is quenched by PA in a concentration-dependent mode (C. A. Baker, K. Desrosiers & J. W. Dolan, unpublished). The change in fluorescence at high PA concentrations suggests that propranolol is acting by binding to and sequestering PA, making the PA unavailable for subsequent activity by LPP, PI4P5K or any other downstream target. Furthermore, when DAG levels were measured in wild-type *C. albicans* cells in response to millimolar concentrations of the drug, total cellular DAG levels remained unchanged compared to untreated cells (C. A. Baker, K. Desrosiers & J. W. Dolan, unpublished). These results, taken together, indicate that PA, rather than DAG, is the important molecule derived from PLD1 activity during morphogenesis.

In this study, the data suggest PLD1 is the source of PA required to stimulate PI4P5K during morphogenesis. Our results provide further evidence for a role of PLD1 in polarized growth (Hube *et al.*, 2001; McLain & Dolan, 1997) and a possible role for PI4P5K in this process. As shown in Fig. 2(a), PI4P5K is highly activated when germ tubes begin to emerge from pre-existing

cells, whereas PLD1 activity does not peak until later. This timing would suggest that PIP_2 formed by the action of PI4P5K stimulates PLD1. There is, however, a highly reproducible 10% increase in PLD1 activity 10 min after induction of morphogenesis before any germ tubes have emerged (Fig. 2a; McLain & Dolan, 1997). It is possible that PA is generated by stimulation of a highly localized pool of PLD1 to initially activate PI4P5K. Supporting this idea, PI4P5K activity was attenuated in extracts prepared from *pld1* Δ null mutant cells and by the addition of propranolol. Interestingly, germ-tube formation in *pld1* Δ cells was unaffected by the lack of PLD1 signalling and PI4P5K stimulation. Similarly, germ tubes were formed normally in response to induction by both serum and temperature, even though PI4P5K activity was relatively unaffected. These results were not unexpected, due to the multiple pathways that can induce hyphal development (Ernst, 2000). Thus, PIP_2 may be important to morphogenesis in response to some stimuli and irrelevant in response to other stimuli. Further studies are needed to determine whether the increase in PI4P5K activity is a cause or consequence of temperature-induced morphogenesis.

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