The role and relevance of phospholipase D1 during growth and dimorphism of *Candida albicans*

Bernhard Hube,^{1,3} Daniela Hess,¹ Carol A. Baker,² Martin Schaller,⁴ Wilhelm Schäfer¹ and Joseph W. Dolan²

Author for correspondence: Bernhard Hube. Tel: +49 1 888 754 2116. Fax: +49 1 888 754 2328.
e-mail: HubeB@rki.de

¹ Institut für Allgemeine Botanik, AMP III, Universität Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany

² Medical University of South Carolina, Department of Microbiology and Immunology, PO Box 250504, Charleston, SC 29425, USA

³ Robert Koch-Institut, NG4, Nordufer 20, D-13353 Berlin, Germany

⁴ Dermatologische Klinik und Poliklinik der Ludwig-Maximilians-Universität München, Frauenlobstr. 9-11, D-80337 München, Germany

The phosphatidylcholine-specific phospholipase D1 (PLD1) in *Saccharomyces cerevisiae* is involved in vesicle transport and is essential for sporulation. The gene encoding the homologous phospholipase D1 from *Candida albicans* (PLD1) was used to study the role of PLD1 in this pathogenic fungus. *In vitro* and *in vivo* expression studies using Northern blots and reverse transcriptase-PCR showed low PLD1 mRNA levels in defined media supporting yeast growth and during experimental infection, while enhanced levels of PLD1 transcripts were detected during the yeast to hyphal transition. To study the relevance of PLD1 during yeast and hyphal growth, an essential part of the gene was deleted in both alleles of two isogenic strains. *In vitro* PLD1 activity assays showed that *pld1* mutants produced no detectable levels of phosphatidic acid, the hydrolytic product of PLD1 activity, and strongly reduced levels of diacylglycerol, the product of lipid phosphate phosphohydrolase, suggesting no or a negligible background PLD1 activity in the *pld1* mutants. The *pld1* mutants showed no growth differences compared to the parental wild-type in liquid complex and minimal media, independent of the growth temperature. In addition, growth rates of *pld1* mutants in media with protein as the sole source of nitrogen were similar to growth rates of the wild-type, indicating that secretion of proteinases was not reduced. Chlamydospore formation was normal in *pld1* mutants. When germ tube formation was induced in liquid media, *pld1* mutants showed similar rates of yeast to hyphal transition compared to the wild-type. However, no hyphae formation was observed on solid Spider medium, and cell growth on cornmeal/Tween 80 medium indicated aberrant morphogenesis. In addition, *pld1* mutants growing on solid media had an attenuated ability to invade the agar. In a model of oral candidosis, *pld1* mutants showed no attenuation of virulence. In contrast, the mutant was less virulent in two different mouse models. These data suggest that PLD1 is not essential for growth and oral infections. However, they also suggest that a prominent part of the phosphatidic acid and diacylglycerol pools is produced by PLD1 and that the level of these components is important for morphological transitions under certain conditions in *C. albicans*.

Keywords: PLD1, virulence, signalling pathways, diacylglycerol, phosphatidic acid

INTRODUCTION

In *Saccharomyces cerevisiae* a phosphatidylcholine

Abbreviations: DAG, diacylglycerol; FOA, 5-fluoroorotic acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PIP, phosphatidylinositol transfer protein; PLD, phospholipase D; RHE, reconstituted human epithelium; RT, reverse transcriptase.

(PC)-specific phospholipase D1 (ScPLD1), located in the particulate fraction of haploid and diploid cells (Ella *et al.*, 1995), was found to be essential for sporulation (Rose *et al.*, 1995). This phospholipase had enhanced activity in media with non-fermentable carbon sources and was able to transfer phosphatidate to primary alcohols (Ella *et al.*, 1995). In 1996, a gene encoding a phosphatidylcholine (PC)-specific phospholipase D

(*SPO14*) was cloned (Ella *et al.*, 1996; Rose *et al.*, 1995; Waksman *et al.*, 1996). Although *spo14* mutants were viable, they were unable to sporulate. ScPLD1-deficient mutants were also deficient in growth on non-fermentable carbon sources (Ella *et al.*, 1996; Waksman *et al.*, 1996) and it was postulated that ScPLD1 must play a role in growth, secretion and regulation of membrane lipid synthesis in yeast (Sreenivas *et al.*, 1998; Xie *et al.*, 1998). Both the activity and the proper localization of the enzyme were needed to form prospore walls during sporulation (Rudge *et al.*, 1998). In addition, it was suggested that ScPLD1 generates a pool of phosphatidic acid (PA) or diacylglycerol (DAG) necessary for budding of nascent secretory vesicles from the *trans*-Golgi network (Xie *et al.*, 1998). It is likely that ScPLD1 plays a dual role in providing lipid components such as PA or DAG, which are necessary for the formation of membranes and secretory vesicles, and may also act as signalling molecules (Rudge *et al.*, 1998). A third potential second messenger, lysophosphatidic acid (LPA), may be produced by the sequential activity of ScPLD1 and a phospholipase A, and may also play important roles in yeast signalling pathways.

Candida albicans is a polymorphic yeast of increasing medical importance. Approximately 90% of all genes found in *C. albicans* have a homologous counterpart in the closely related yeast *S. cerevisiae* (Magee & Scherer, 1998). However, the function of these homologous genes may have changed because *C. albicans* has long been adapted to growth in the human host. For example, genes encoding components of the MAP kinase mating pathway in *S. cerevisiae* have homologous counterparts in *C. albicans*. However, in *C. albicans* this pathway regulates the yeast to hyphal transition (dimorphism), one of the important virulence attributes of *C. albicans* (Brown & Gow, 1999; Ernst, 2000). In addition to this pathway, which terminates with the transcriptional activator Cph1 (Liu *et al.*, 1994), at least two additional morphogenetic pathways exist. One of these additional pathways terminates with the transcriptional regulator Efg1 (Stoldt *et al.*, 1997) while the remaining pathway terminates with the transcriptional repressor Tup1 (Braun & Johnson, 1997). Furthermore, changes in the level of the intracellular second messenger cAMP play an important role in the regulation of dimorphism (Brown & Gow, 1999; Ernst, 2000).

Since *SPO14* is essential for sporulation in *S. cerevisiae* and sporulation of *C. albicans* has yet to be observed, a homologous *PLD1* gene may have a different function in the pathogenic yeast. In fact, a PC-specific PLD activity providing PA, DAG and/or LPA, was shown to be involved in the yeast to hyphal transition (McLain & Dolan, 1997). The transition was stimulated by the addition of exogenous PLD. Furthermore, the addition of 1-propanol, which resulted in the production of phosphatidylpropanol by PLD1 at the expense of the usual product, phosphatidic acid, delayed hyphal formation. These data indicated that *C. albicans* possessed a *PLD1* gene and that, in addition to the known protein kinase-based signalling pathways, lipid molecules may

be involved in the regulation of the dimorphic transition of *C. albicans*. The aim of this study was, therefore, to study the role and relevance of the gene encoding PLD1 during growth and dimorphism of *C. albicans*.

METHODS

***C. albicans* and *Escherichia coli* strains.** The Ura⁻ strain CAI4 of *C. albicans* (Robin) Berkhout, used for disruption of *PLD1*, and the *ura3::imm434* Ura⁺ (heterozygote of CAI4) CAF2-1 were kindly provided by Dr W. A. Fonzi, University of Washington, USA. Growth rates, dimorphism and phospholipase assays of *pld1::hisG* Ura⁺ isogenic mutants were compared with the parental Ura⁺ clinical isolate SC5314 (Gillum *et al.*, 1984), the heterozygote CAF2-1, and/or the morphogenic mutants *cph1::hisG* Ura⁺, *efg1::hisG* Ura⁺ and *cph1::hisG/efg1::hisG* Ura⁺ (kindly provided by Dr G. R. Fink, Massachusetts Institute of Technology, USA and Dr J. Ernst, University of Düsseldorf, Germany). In addition, strain B311 (Vasquez-Torres *et al.*, 1999) was used for virulence studies. *E. coli* XL-Blue (Stratagene) was used for transformation of cloned *PLD1* fragments.

Plasmids and fosmid library. pBluescript KS (+/-) (Stratagene) was used for subcloning of *PLD1* fragments. The fosmid library (Magee & Scherer, 1998) was kindly provided by Dr S. Scherer, University of Minnesota, USA.

Media and growth conditions. To investigate *PLD1* gene expression in SC5314 and growth or hyphal formation of *pld1::hisG* Ura⁺ mutants, we used liquid YPG, minimal and protein medium (Hube *et al.*, 1994). For hyphal growth, cells were suspended in 5% (v/v) calf serum (Gow & Gooday, 1982), or induced by the addition of *N*-acetylglucosamine (Mattia *et al.*, 1982) or the regime of pH/temperature-regulated yeast to hyphal transition was used (Buffo *et al.*, 1984). To perform growth tests on solid media, *C. albicans* cells were grown overnight in YPG and diluted to 2×10^7 cells ml⁻¹. Five microlitres of this suspension and 5 µl serial dilutions of each strain were spotted onto each type of plate (YPG, minimal medium, protein medium), and the plates were incubated at 25 °C, 37 °C and 42 °C for at least 2 d. For hyphal induction on solid media, cells were streaked onto Spider agar (Liu *et al.*, 1994) and cornmeal agar with 1% Tween 80 (CMA/T) (Buckley *et al.*, 1982) and incubated at 37 °C. For chlamydospore formation, cells were streaked on Spider agar or CMA/T at low concentrations, covered with coverslips and incubated at 25 °C for several days. Colonies were photographed using a Zeiss KF2 light microscope equipped with a Polaroid MicroCam camera. Pictures of representative colonies were taken after 24 h on CMA/T and after 72 h on Spider agar. Following photography, the plates were washed vigorously with water to remove cells on the surface of the agar. The plates were allowed to dry at room temperature for 1 h prior to the second round of photography.

PCR. PCR was used to prepare the disruption cassette and hybridization probes for Southern and Northern blots, and to confirm the disruption of the *PLD1* gene. The following pairs of primers were used to amplify hybridization probes of the ORF of *SPO14*: ScPLD1-1 (5'-TGTCGTTATTGATGAAACAT-3') and ScPLD1-2 (5'-TTATCAAGTCGGTGTCTCTA-3'); ScPLD1-3 (5'-CATCAATAACGACAACTTCTC-G-3') and ScPLD1-4 (5'-CGTCGCGTTCCAACAACCTCAC-3'); ScPLD1-5 (5'-TAGAGACACCGACTTGATAAAG-3'); and ScPLD1-6 (5'-TCCAGTGAACCATCATCTAG-3'). The following primers were used to amplify fragments of the ORF of *PLD1*: PLD1-1 (5'-GACCAACGCATACCAATTC-3');

PLD1-2 (5'-CAGCTTGTTCATCGACGG-3'); PLD1-3 (5'-AGCTGCCATATATGGCTTACC-3'); PLD1-4 (5'-GACAGCACTAAGAGTGGCAG-3'); PLD1-5 (5'-GAATGAGGT-TGATGAGAGAGC-3'); PLD1-6 (5'-CAAAGATACAGTA-GGGAAGTC-3').

RNA isolation. For Northern analysis, total RNA from *C. albicans* was prepared as described by Hube *et al.* (1994). For reverse transcriptase (RT)-PCR, total RNA was isolated using RNAPure (PepLab Biotechnologie) according to the manufacturer's instructions.

Northern and Southern analyses. Northern blot analysis was performed as described by Hube *et al.* (1994). For Southern blot analysis of genomic or fosmid DNA, standard protocols were used (Sambrook *et al.*, 1989). Southern blots and the fosmid library were hybridized to identify *PLD1* using a non-radioactive digoxigenin (DIG)-labelling kit (Boehringer Mannheim). Three PCR-generated probes of *SPO14* were used. Probe A was amplified with ScPLD1-1 and ScPLD1-2, and contained a 1000 bp fragment of the *SPO14* ORF encoding the conserved boxes 4, 5 and 7 (Waksman *et al.*, 1996). Probe B was amplified with ScPLD1-3 and ScPLD1-4, and contained a 1100 bp *SPO14* fragment located 5' of probe A, and probe C was amplified with ScPLD1-5 and ScPLD1-6, and contained a 1380 bp *SPO14* fragment located 3' of probe A. To analyse *PLD1* gene disruption, a 430 bp long T7 (Stratagene) *PLD1*-2 PCR fragment of plasmid pHPLD1 or the random labelled *hisG::URA3::hisG* cassette of pMB7 was used as probe. For Northern blots, the same fragment of subclone pHPLD1 and a 700 bp PCR fragment of *TEF3* (Colthurst *et al.*, 1992; Hube *et al.*, 1994) were labelled with [α - 32 P]dCTP (~3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) (Amersham). mRNA levels were measured relative to the rRNAs by loading approximately equal amounts of total RNA in each lane of the Northern blots. In addition, the *TEF3* mRNA (Colthurst *et al.*, 1992) was probed as a positive (non-quantitative) control (Hube *et al.*, 1994).

For Southern blots, membranes were hybridized without formamide using digoxigenin-labelled probes and washed as described by the manufacturer (Boehringer Mannheim) with either 65 °C (low stringency) or 68 °C (high stringency) incubation temperatures.

Screening of the fosmid library. The fosmid library was blotted onto nylon membranes using a vacuum dot-blot system (Schleicher & Schuell). *E. coli* cells were lysed and fosmid DNA was denatured with 0.5 M NaOH, 1.5 M NaCl. After neutralization with 1 M Tris/HCl pH 8.0, 1.5 M NaCl, fosmid DNA was fixed by baking and membranes were hybridized as described for Southern analysis. DNA from positive fosmids was isolated as described by Hube *et al.* (2000).

DNA sequencing. DNA subcloned into pBluescript was sequenced by SeqLab Laboratories or MWG.

RT-PCR. One microgram of DNase I-treated total RNA was used for cDNA synthesis as described by Schaller *et al.* (1998). The cDNA was purified using NucleoSpin extract columns (Macherey & Nagel). To detect *PLD1* transcripts, primers PLD1-5 and PLD1-6 were used to amplify an 889 bp cDNA fragment of *PLD1*. To prove the absence of contaminating genomic DNA, we used primers specific for the intron-containing gene encoding elongation factor 1 (*EFB1*) (Maneu *et al.*, 1996). Using primers EFB5' (5'-ATTGAACGAATTC-TTGGCTGAC-3') and EFB3' (5'-CATCTTCTTCAACAG-CAGCTTG-3'), a 916 bp PCR fragment was amplified when

genomic DNA was present. In contrast, a 551 bp sized RT-PCR fragment of the *EFB1* transcript which does not contain an intron of 365 bp in size was amplified when cDNA was used as a template. Even traces of DNA were detectable, when EFB5' and the intron-specific primer EFBint (5'-TCTTGAG-GCCACCTCATAAAC-3') were used. In these control experiments an additional 264 bp fragment was amplified.

Candida transformation and gene disruption. Protoplasts were prepared and *PLD1* disruption cassettes were transformed as described by Hube *et al.* (1997). For 5-fluoroorotic acid (FOA) selection of Ura⁻ recombinants (Fonzi & Irwin, 1993; Gow *et al.*, 1994), cloned transformants were resuspended in 1 ml water and plated on SD agar containing FOA (0.01 g ml⁻¹) and uridine (25 µg ml⁻¹).

To construct a disruption cassette, pHPLD1 (Fig. 1b) was digested with *SacI* and *SalI*, which removes a 2075 bp internal fragment. Since this fragment was 450 bp longer than expected from the published sequence (Kano *et al.*, 1998), we sequenced this part of the gene and found a T instead of a C in position 1266, which created a second *SacI* site at 1262 bp. The removed fragment contained sequences encoding the HXKXXXXD, HKD and GGGR motifs, which are critical for activity of ScPLD1 in *S. cerevisiae* (Waksman *et al.*, 1996). The *hisG::URA3::hisG* cassette of pMB7 (Fonzi & Irwin, 1993) was removed by *SacI*/*SalI* digestion, and ligated into pHPLD1 to give pHPlura (Fig. 1b). pHPlura was linearized with *PvuII* and transformed into CAI4. Integration of the cassette into the *PLD1* locus was confirmed by Southern analysis for each step of the disruption procedure (Fig. 1c, d). When genomic DNAs of first round transformants were digested with *EcoRI* and hybridized to the *PLD1* probe, wild-type alleles were >12 kb in size (Fig. 1c). Alleles which contained the *hisG::URA3::hisG* cassette showed two bands, one with a size similar to the wild-type band and a second band of 3.5 kb. The correct integration could be confirmed using *BamHI*, which again produced a second band of 3.5 kb in addition to the large band when hybridized to the *PLD1* probe. *EcoRI* and *BamHI* digests of DNA from FOA-resistant segregants showed a single band 1 kb smaller than the size of the wild-type band when hybridized with the *PLD1* probe. Since it was difficult to distinguish between the wild-type band and the large bands of disrupted alleles, Southern analysis was repeated using a *hisG::URA3* probe to confirm the disruption of both alleles (Fig. 1d). In first round transformants a 9 kb and a 3.5 kb fragment of the disrupted allele hybridized in *EcoRI* digests as expected. In FOA segregants only one band >9 kb containing the remaining *hisG* part hybridized. In second round transformants the larger band >9 kb, the 9 kb band and the 3.5 *EcoRI* band were all visible, confirming that both alleles were disrupted and no additional integration had occurred. Using the two primers pHPLD1-3 and pHPLD1-4, *hisG* integration and disruption of both alleles in two isogenic strains was confirmed by PCR (not shown). The two isogenic *pld1::hisG/pld1::hisG::URA3::hisG* null mutants (Ura⁺) were named *pld1Δ1* and *pld1Δ2*, and used for growth and activity tests.

PLD1 assays. Cell extracts were prepared and PLD1 activity was assayed as described by McLain & Dolan (1997).

Experimental infection. Virulence properties of *pld1* mutants were tested in an *in vitro* model of oral candidosis based on reconstituted human epithelium (RHE) as described by Schaller *et al.* (1998). RHE was infected with 2 × 10⁶ *C. albicans* wild-type or mutant cells in 50 µl PBS. Controls contained 50 µl PBS alone. Inoculated and uninoculated

Fig. 1. Restriction map of the genomic locus of *PLD1* (a), the construction of pHP1ura (b) and Southern analysis of *PLD1* transformants (c, d). The 5130 bp ORF of *PLD1* (dotted box) and important restriction sites are shown in (a). The *PstI* fragment was cloned in pHP1ura and used to produce the disruption cassette. The HXXXXXXD, HKD and GGGR motifs (small black boxes) are located between the *SalI* and the second *SacI* site of the ORF. An additional *SacI* site was identified at the 5' end. This essential *SacI*-*SalI* fragment was replaced by the *hisG::URA3::hisG* cassette introducing a new *EcoRI* site in the disrupted ORF. Restriction digests of the wild-type CAI4, the first transformant, first FOA-segregant and second transformant (*pld1Δ1*) were hybridized with the *PLD1* (c) or the *hisG::URA3* probe (d). The same results were obtained for a second isogenic mutant (*pld1Δ2*). See text for further details.

cultures were incubated at 37 °C with 5% CO₂ at 100% humidity for 12–48 h.

Animal studies. Germ-free immunodeficient beige nude (*bg/bg nu/nu*) mice (Fodstad *et al.*, 1984) were inoculated orally with approximately 1×10^6 c.f.u. of either wild-type SC5314 or *pld1Δ1* mutant cells; a second wild-type strain, B311, was also used for comparison (Vasquez-Torres *et al.*, 1999). Viability was monitored for 6 weeks post-inoculation. Germ-free immunodeficient transgenic ε26 mice (Wang *et al.*, 1994) were inoculated orally with approximately 1×10^6 c.f.u. wild-type SC5314, wild-type B311 or *pld1Δ1* mutant cells. Viability was again monitored for 6 weeks post-inoculation. Survival curves were calculated according to the method of Kaplan-Meier using GraphPad Prism 3.0a software and were compared using the log rank test of Mantel-Haenszel.

RESULTS

Cloning of *PLD1*

To identify genes in *C. albicans* homologous to *SPO14*, genomic DNA of SC5314 was digested with *EcoRI* and blotted onto membranes. The blot was hybridized with *SPO14* probes and four distinct bands between 3 and 10 kb were identified, indicating that homologous sequences exist in the genome of *C. albicans*. The same probes were used to screen 10 membranes of a fosmid library, each containing 96 fosmid clones (Magee & Scherer, 1998). Two positive clones, 2G2 and 6E4 (<http://alces.med.umn.edu/candida/fosmidinfo.html>) were further analysed. Each clone contained a 7.5 kb

PstI and a 9.0 kb *EcoRI* fragment. The 7.5 kb fragment was cloned into the *PstI* site of pBluescript KS (+/–) to give pHP1ura. Sequencing from both ends revealed ORFs. Sequences from the T3 site had homologies to several hypothetical GTPase genes with the highest similarity to an ORF of *S. cerevisiae* with unknown function (accession no. U51032). The opposite strand of the T7 site encoded an amino acid sequence with high similarity (40% identity) to ScPLD1 corresponding to amino acids 473–826 of the deduced protein. The DNA sequence of this region of the *PstI* fragment was found to be 98% identical to the *PLD1* sequence published by Kanoh *et al.* (1998) and localized on contig numbers Con4-1904 and Con4-2834 of the Stanford genome project website (<http://www-sequence.stanford.edu/group/candida/index.html>; gnl|Stanford-5476|C.albicans-Con4-1904 Candida albicans unf... 7002 0.0; gnl|Stanford-5476|C.albicans-Con4-2834 Candida albicans unf... 3251). Since fosmid 6E4 was mapped to chromosome 1, *PLD1* must be located on this chromosome.

PLD1 in vitro expression studies

Since *PLD1* activity was stimulated by inducers of the dimorphic transition (McLain & Dolan, 1997), we questioned whether *PLD1* is regulated during morphogenesis. Hyphal formation of *C. albicans* was induced by pH/temperature shift in Lee's medium (Buffo *et al.*, 1984). Cell samples were taken 60, 90, 120, 180

Fig. 2. Expression of *PLD1* during yeast to hyphal transition in *C. albicans* shown by Northern analysis (a) and RT-PCR (b, c). Total RNA of pH/temperature induced cells was analysed in (a) and (b). Expression of *PLD1* in *N*-acetylglucosamine induced cells is shown in (c). The housekeeping genes *TEF3* and *EFB1* were used as internal controls (Hube *et al.*, 1994; Schaller *et al.*, 1998). mRNA levels were measured relative to the rRNAs by loading approximately equal amounts of total RNA in each lane of the Northern blots. In addition, the *TEF3* mRNA (Colthurst *et al.*, 1992) was probed as a positive (non-quantitative) control (Hube *et al.*, 1994). For RT-PCR, the *EFB1* primers amplifying the intron-containing region were used to show the absence of contaminating DNA. *EFB1* cDNA revealed a 551 bp fragment. Only traces of DNA were detectable when primer EFB5' and the intron-specific primer EFBint were used. In these control experiments an additional 264 bp fragment was amplified when DNA was present.

and 300 min after induction, hyphal formation was monitored and total RNA was isolated. After 60 min, no germ tube formation was seen; after 90 min, germ tube formation was 57%, 83% after 120 min and >90% after 180 min. The RNA was used to measure *PLD1* transcripts by Northern analysis and RT-PCR (Fig. 2). Northern analysis showed expression of *PLD1* at 180 min, but no signals in the other samples (Fig. 2a). In contrast, the more sensitive RT-PCR analysis showed a continuous up-regulation of *PLD1* expression during the dimorphic transition (Fig. 2b). A similar expression pattern was seen when the experiment was repeated. To show that the up-regulation was due to the morphological transition, hyphal formation was also induced by the addition of *N*-acetylglucosamine (Mattia *et al.*, 1982). After 120 min, 42% of the cells produced germ tubes. After 150 and 180 min, 68% and 85% of the cells

Fig. 3. Continuous low level expression of *PLD1* during growth in medium containing BSA as the sole source of nitrogen as determined by RT-PCR.

showed hyphal formation. Expression of *PLD1* was observed during the course of germ tube production (Fig. 2c). Similar results were obtained when this experiment was repeated or when hyphal formation was induced by the addition of serum (not shown).

In *S. cerevisiae* it has been postulated that ScPLD1 may play a role in growth, secretion and regulation of lipid biosynthesis (Sreenivas *et al.*, 1998). Therefore, we studied the expression of *PLD1* in medium with protein as the sole source of nitrogen. Under these conditions high levels of secreted aspartic proteinases must be secreted to support growth, while cells unable to secrete distinct Saps do not grow (Hube *et al.*, 1997). *C. albicans* cells were grown in protein medium and *PLD1* expression was monitored 30, 60, 120, 180 and 300 min after inoculation using RT-PCR (Fig. 3). A continuous low-level expression of *PLD1* was observed in two independent experiments. In addition, YPD precultures without proteinase-inducing BSA also showed *PLD1* expression (not shown).

PLD1 is expressed during experimental infections

To investigate whether *PLD1* is expressed during infections with *C. albicans*, we screened for mRNA transcripts in infected artificial tissue. A model of oral candidosis based on RHE (Schaller *et al.*, 1998) was used to study *PLD1* expression during experimental infection. Total RNA was isolated from infected RHE at different time points during the course of an infection and mRNA transcripts were amplified by RT-PCR using primers PLD1-5 and PLD1-6. *PLD1* transcripts were detectable 12, 36 and 48 h after infection, indicating that *PLD1* is expressed during an infection process.

PLD1 encodes a phospholipase D

Expression studies showed a low-level expression of *PLD1* under most conditions and an up-regulation during hyphal formation. *PLD1* activity has also been shown to increase during morphogenesis (McLain & Dolan, 1997). To investigate the relevance of *PLD1* during growth and dimorphism, we produced mutants that lack functional copies of *PLD1* (Fig. 1). In these

Fig. 4. *pld1* null mutants lack detectable PLD1 activity. Cell extracts from the wild-type (CAF2-1), *pld1Δ1* and *pld1Δ2* strains were assayed for PLD1 activity with the fluorescent *in vitro* assay (McLain & Dolan, 1997). The sample in lane 1 contained no cell extract; lane 2 contained cell extract from *efg1*; lane 3 contained cell extract from *cph1*; lane 4 contained cell extract from *efg1/cph1*; lane 5 contained cell extract from wild-type strain CAF2-1; lane 6 contained cell extract from *pld1Δ1* and lane 7 contained cell extract from *pld1Δ2*. BPC, BODIPY-phosphatidylcholine; BPA, BODIPY-phosphatidic acid; BDG, BODIPY-diglyceride.

mutants, an essential part of each allele of the *PLD1* gene was removed in two isogenic strains.

Sequence alignment of *PLD1* with *SPO14* and other phospholipase D genes strongly suggested that *PLD1* encodes a phospholipase D. Southern analysis of wild-type and *pld1* null mutants and sequence comparison with sequence data from the *Candida* genome project (<http://www-sequence.stanford.edu/group/candida>) suggested that *PLD1* exists as a single copy gene and that *C. albicans* does not contain additional homologous *PLD* genes. However, additional heterologous *PLD* genes may exist in *S. cerevisiae* (Mayr *et al.*, 1996; Waksman *et al.*, 1997). To prove whether *PLD1* encodes phospholipase D1 and whether this is the only gene responsible for PLD1 activity in *C. albicans*, we measured PLD activity using a fluorescent analogue of PC (BODIPY-phosphatidylcholine; Molecular Probes) in extracts from wild-type and the *pld1* null mutants. No detectable BODIPY-phosphatidic acid and strongly reduced levels of BODIPY-diglyceride were detected in both *pld1Δ1* and *pld1Δ2* when compared with the wild-type SC5314 or CAF2-1 (Fig. 4), indicating that *PLD1* encodes the major or only PC-specific phospholipase D in *C. albicans*. Fig. 4 also demonstrates that basal PLD1 activity is unaffected by mutations in *CPH1*, *EFG1* or both.

Growth of *pld1* null mutants

To test the effect of the lack of PLD1 activity on cell growth of *C. albicans*, several growth conditions were tested. Since expression of *PLD1* was observed in complex media, we first analysed the growth of *pld1Δ1* and *pld1Δ2* in YPG medium. No differences in growth rates were observed at 25 °C, 37 °C or 42 °C. The same results were observed when the *pld1* mutants were grown in minimal medium with protein as the sole source of nitrogen at 37 °C, indicating that sufficient proteinases were secreted and secretion was not reduced significantly due to the lack of PLD1 activity.

Hyphal formation of *pld1* null mutants in liquid media

Expression of *PLD1* was observed during the yeast to hyphal transition when cells were induced by pH/temperature shift, *N*-acetylglucosamine or serum. To study the relevance of PLD1 during the dimorphic transition, the *pld1* mutants were induced and the rate of hyphal formation was compared with the wild-type strain SC5314. No differences were observed in all cases. For example, in serum-induced cultures both wild-type and mutant cells had more than 50 % germ tubes after 60 min and more than 90 % hyphal cells after 120 min.

Chlamydospore production

C. albicans has the ability to form thick-walled cells, termed chlamydospores, which arise on elongated suspensor cells situated on pseudohyphal or hyphal cells. Although they do not appear to allow long term survival, the morphology resembles true yeast spores. *S. cerevisiae spo14* mutants were viable, but unable to sporulate. Therefore, we investigated the role of PLD1 during chlamydospore formation. Chlamydospore formation was induced on Spider agar and CMA/T under micro-aerobic conditions at 25 °C. Although suspensor cells of *pld1* mutants sometimes appear to be more swollen compared to wild-type suspensor cells, both *pld1* mutants were able to produce chlamydospores to the same extent as the wild-type.

Morphological defects of *pld1* null mutants on solid media

Although no differences in hyphal formation were seen in liquid media, there were substantial differences on solid media. On solid serum agar, an overall reduction of hyphal formation was seen for the *pld1* mutants when compared to the wild-type. On Spider agar, *pld1* mutants failed to make hyphae that radiate away from the central colony, a phenotype previously reported for *cph1* (Liu *et al.*, 1994) and *efg1* (Stoldt *et al.*, 1997) mutants (Fig. 5). The *pld1* mutants produced hyphae that penetrated into the agar under the colony, but the extent of penetration was reduced relative to that seen with the wild-type or *cph1* mutant. The hyphal formation of the wild-type is variable from one plate to another, making absolute quantitations impossible. Nevertheless, the majority (greater than 50 %) of wild-type colonies formed radiating hyphae on Spider medium while the *pld1* mutants failed to form any colonies with radiating hyphae. The phenotype on CMA/T medium was most pronounced after the first 24 h growth. The number of hyphae radiating from the *pld1* mutant colonies (Fig. 5h, i) was substantially greater than the number radiating from either the wild-type (Fig. 5g) or the *cph1* mutant (Fig. 5j). As with Spider medium, the wild-type colonies exhibited some degree of variability. Nevertheless, greater than 75 % of wild-type colonies gave rise to fewer than 10 radiating hyphae after 24 h at 37 °C with most producing only one or two

Fig. 5. Phenotype of mutants on inducing media. Cells were streaked onto Spider agar (a–f) and CMA/T (g–l) and incubated at 37 °C. Strains used were CAF2-1 (a, g), *pld1Δ1* (b, h), *pld1Δ2* (c, i), *cph1* (d, j), *efg1* (e, k) and *cph1/efg1* double mutant (f, l). Colonies on Spider agar were magnified 20× and the colonies on CMA/T were magnified 80×. The arrowheads in (g) indicate kinks in hyphae that are common to wild-type hyphae but essentially absent from the *pld1* hyphae. Bars, 1 mm (f); 0.2 mm (l). Parts (a–f) and (g–l) have the same magnifications.

such hyphae. In contrast, all *pld1* colonies gave rise to numerous hyphae (20–50 hyphae per colony) radiating from the central colony. Furthermore, the hyphae produced by the *pld1* mutants were straighter and shorter than those produced by the wild-type or *cph1* colonies, which were long and kinked (marked with arrowheads in Fig. 5).

S. cerevisiae cells exhibit a haploid-specific ability to invade agar and this process is regulated by components of the pheromone response pathway (Roberts & Fink, 1994; Cook *et al.*, 1996). Homologues of some of these components regulate morphogenesis in *C. albicans*; therefore, the ability of wild-type and *pld1* mutant cells to invade the agar was also examined (Fig. 6). Cells within the agar more clearly revealed the differences in the ability of the strains to invade a solid surface and in the pattern of lateral budding along the hyphae. The wild-type cells and the *cph1* mutant robustly invaded the agar of Spider medium, producing hyphae that were heavily decorated with yeast-form cells (Fig. 6a, c). The *efg1* mutant cells had a reduced ability to penetrate the surface of Spider medium and failed to form hyphae of any substantial length (Fig. 6d). The *pld1* mutants were able to invade Spider agar, but failed to form lengthy

Fig. 6. Mutants have a distinct agar-invasion phenotype. Washed plates were photographed to examine the morphologies of hyphae within the agar medium. (a–d) Cells on Spider agar; (e–h) cells on CMA/T. The strains examined were CAF2-1 (a, e), *pld1Δ1* (b, f), *cph1* (c, g) and *efg1* (d, h). The arrowheads in (e) indicate hyphae decorated with large clusters of cells that are common to wild-type hyphae but essentially absent from the *pld1* hyphae. Bars, 1 mm (d); 0.2 mm (h). Parts (a–d) and (e–h) have the same magnifications.

hyphae and the hyphae that did form were not as heavily decorated by yeast-form cells (Fig. 6b). Differences in lateral budding were also seen with cells growing on cornmeal agar in which wild-type and *cph1* cells formed clumps of yeast cells at regular intervals along the hyphae (Fig. 6e, g). The *pld1* mutants formed small clusters containing only a few cells per cluster (Fig. 6f). As with Spider medium, the *efg1* mutant cells were not able to invade the agar efficiently and produced only foci of cells just below the surface (Fig. 6h). The relevance of these defects to growth *in vivo* is not known at this time.

Null mutants exhibit reduced virulence

Since we showed that the *PLD1* gene is expressed in wild-type strains during infection of our model of oral candidosis (RHE), we infected the RHE tissue with the *pld1* null mutants and compared the phenotype with the parental wild-type strain. Histological samples were taken 12 h and 36 h after infection and 60 sections were analysed for each time point and each strain. No obvious differences were observed between the wild-type and mutant strains.

Mutations in several genes regulating morphogenesis in *C. albicans* share the same phenotype on solid Spider medium as the *pld1* mutants. These other mutants also exhibit reduced virulence in animal models of infection compared to the parental strain SC5314. The lethality of the *pld1Δ1* mutant was assessed in a mouse model utilizing *bg/bg nu/nu* immunodeficient mice (Fodstad *et al.*, 1984). For this experiment, an additional wild-type strain of *C. albicans* was used. B311 and SC5314 are independently isolated wild-type strains. Strain B311 has been used extensively in animal studies, including studies with the *bg/bg nu/nu* mice, and was included solely to provide a point of reference to earlier virulence studies with this strain of mice. The survival of mice orally inoculated with 10^6 c.f.u. was followed for up to

Table 1. Lethality of *C. albicans* strains in *bg/bg nu/nu* mice

Mice were inoculated orally with 1×10^6 c.f.u. of the indicated strain of *C. albicans*. Differences between wild-type and *pld1Δ1* curves were significant with *P* values of 0.0002 (B322 vs. *pld1Δ1*) and 0.0027 (SC5214 vs. *pld1Δ1*).

Candida strain	Time post-inoculation (d)	Survival	
		Percentage	No. alive/no. dead
B311	14	100	12/0
	21–28	58	7/5
	35–42	0	0/12
SC5314	14	100	4/0
	21–28	0	0/4
	35–42	0	0/4
<i>pld1Δ1</i>	14	100	6/0
	21–28	100	6/0
	35–42	100	6/0

Table 2. Lethality of *C. albicans* strains in *tgε26* mice

Mice were inoculated orally with 1×10^6 c.f.u. of the indicated strain of *C. albicans*. Differences between wild-type and *pld1Δ1* curves were significant with *P* values of <0.0001 (B311 vs. *pld1Δ1*) and 0.0016 (CAF2-1 vs. *pld1Δ1*).

Candida strain	Time post-inoculation (d)	Survival	
		Percentage	No. alive/no. dead
B311	14	100	17/0
	21–28	18	3/14
	35–42	0	0/17
CAF2-1	14	0	0/5
	21–28	0	0/5
	35–42	0	0/5
<i>pld1Δ1</i>	14	100	6/0
	21–28	100	6/0
	35–42	100	6/0

42 d post-inoculation (Table 1). As expected, both wild-type strains of *Candida* were 100% lethal to the immunodeficient mice with the death of all inoculated animals within 42 d post-inoculation. In contrast, all animals inoculated with *pld1Δ1* cells survived to 42 d post-inoculation and were healthy when killed.

The ability of cells lacking PLD1 activity to produce a lethal infection was also determined using transgenic epsilon 26 (*tgε26*) mice (Wang *et al.*, 1994). Following oral inoculation, *pld1Δ1* mutant cells were significantly less virulent than wild-type strains of *C. albicans* (Table 2). Both wild-type strains tested (CAF2-1 and B311) were able to establish lethal infections resulting in

mortality by 42 d post-challenge. In contrast, all mice inoculated with the PLD1-deficient yeast were alive and healthy at the end of the experiment. These assessments of virulence in two independent strains of immunodeficient mice indicate that the loss of PLD1 activity may have a profound effect on the ability of the cell to establish a disseminated and lethal infection.

DISCUSSION

One of the most prominent phenotypes of *S. cerevisiae spo14* null mutants was the fact that cells were unable to sporulate (Ella *et al.*, 1996; Rose *et al.*, 1995; Waksman *et al.*, 1996). Since *C. albicans* has not been shown to sporulate, we reasoned that a homologous phospholipase D1 in this fungus may have a different function. Earlier experiments from McLain & Dolan (1997) showed that at least one calcium-independent, phosphatidylinositol 4,5-bisphosphate-stimulated, oleate-sensitive form of PLD1 exists in *C. albicans*. These studies also demonstrated that this enzyme was important to the process of dimorphic transition. Research on the regulation of dimorphism was recently focused on MAP kinases, cAMP-regulated pathways and transcriptional regulators (Brown & Gow, 1999; Ernst, 2000). In contrast, very few studies of the role of lipids as second messengers in the regulation of morphology in *C. albicans* have been reported (Gadd, 1995; Gadd & Foster, 1997). Therefore, the aim of this study was to clone the *PLD1* gene and to investigate the role and importance of this gene during growth and hyphal formation. The gene encoding *PLD1* was cloned from a fosmid library. Sequencing of a major part of the ORF revealed a 98% identity to the sequence published by Kanoh *et al.* (1998). The observed differences may be due to sequencing errors, strain differences or the fact that different alleles were sequenced.

PLD1 is a large gene encoding a protein of 1710 aa with a calculated molecular mass of 196.4 kDa (Kanoh *et al.*, 1998). The overall identity of the deduced protein to ScPLD1 was 42% (Kanoh *et al.*, 1998) with highly conserved HXKXXXXD, HKD and GGGR motifs in the active site.

In this study, we found that *PLD1* indeed showed a higher expression during the yeast to hyphae transition, but also constitutive expression during yeast growth in several media, indicating a more general function of this gene. To study the overall relevance of *PLD1* for growth and during the dimorphic transition in particular, we designed *pld1* null mutants that lacked an essential part of the ORF. Activity assays showed that these mutants were unable to produce PA, the hydrolytic product of PLD1, derived from PC indicating that *PLD1* in fact encodes a phospholipase D. Moreover, these results suggested that *PLD1* is the only or most prominent gene encoding a PC-specific PLD in *C. albicans*.

Null *pld1* mutants were viable on all tested media, indicating that *PLD1* is not an essential gene. Furthermore, no differences in growth rates compared to the wild-type were seen in all liquid media. These

experiments included growth in protein medium, indicating that proteinase secretion was not significantly reduced. In addition, chlamydospore formation was still possible in the *pld1* mutants. In a model of oral infections, no attenuated virulence phenotype was observed. In two different mouse models, however, the *pld1Δ1* mutant was significantly less virulent than wild-type strains.

Therefore, we concluded that PLD1 does not play a major role during growth, chlamydospore formation, secretion in liquid media or oral infections. However, on solid media marked differences were seen, with the lack of hyphal production on Spider medium the most prominent phenotype. Such an inability to form hyphae on Spider medium was also observed for the mutants lacking the transcriptional regulators Cph1 (Liu *et al.*, 1994) or Efg1 (Stoldt *et al.*, 1997). To prove that the observed phenotype was due to the lack of a functional *PLD1* gene and not to undetected mutations introduced during the gene disruption procedure, we produced a second isogenic *PLD1* null mutant (*pld1Δ2*). This second mutant showed the same phenotype as *pld1Δ1* under all conditions, making it unlikely that an undetected mutation was responsible for the observed phenotype.

In principle, PLD1 may act in two different ways during growth and dimorphism. It may produce lipid second messenger molecules which have regulatory functions or simply provide or change lipid molecules necessary as structural material for membranes (or both). For example, PA, LPA and DAG have been demonstrated to enhance membrane curvature (Kearns *et al.*, 1997; Schmidt *et al.*, 1999). Alternatively, PA may interact with proteins to alter cytoskeletal organization. PA has been demonstrated to stimulate the activity of phosphatidylinositol-4-phosphate 5-kinase in both mammals and *S. cerevisiae*; the resulting accumulation of phosphatidylinositol 4,5-bisphosphate can serve as a focal point for the assembly of cytoskeletal proteins containing pleckstrin homology (PH) domains (Lemmon *et al.*, 1996; Tall *et al.*, 1997). In addition, LPA may be generated by the concerted action of PLD1 and a phospholipase A (Mago & Khuller, 1990; Goyal & Khuller, 1992), which may act as an inducer of hyphal formation. LPA is known to be a major extracellular signal, produced in large amounts by activated platelets and other cells in human serum, which activates G proteins (Moolenaar, 1995; Gaits *et al.*, 1997). Since a low molecular mass filtrate of serum was shown to be an inducing agent of the dimorphic transition via the *RAS1* gene product (Feng *et al.*, 1999), LPA may be a candidate signalling molecule which can be produced by host and/or *Candida* cells during infection. Finally, PLD1 may provide DAG, via lipid-phosphate phosphohydrolase, as a signalling molecule or material for a growing hyphal tip. In yeast cells, it has been shown that DAG plays an essential role in vesicular trafficking (Kearns *et al.*, 1997).

A role for PLD1 activity in secretion is supported by work on phosphatidylinositol transfer proteins (PITPs) in *C. albicans* and other yeasts. The only published

regulator protein of fungal PLD1 is an atypical PITP (Li *et al.*, 2000). Although the physiological relevance of this regulation has not been established, this regulation adds to the genetic interaction described between PLD1 and the classic fungal PITP, Sec14p. *SEC14* is an essential gene in *S. cerevisiae* and a number of extragenic '*SEC14* bypass' suppressors have been identified. For these suppressor mutations to bypass the requirement for Sec14, the cell must possess functional PLD1 (Sreenivas *et al.*, 1998; Xie *et al.*, 1998). This suppressor analysis suggests a role for PLD1 in secretion, particularly in the budding of nascent secretory vesicles from the *trans*-Golgi network. Furthermore, work on Sec14 in both *Yarrowia lipolytica* (Lopez *et al.*, 1994) and *C. albicans* (Monteoliva *et al.*, 1996; Riggle *et al.*, 1997) has revealed a possible role for Sec14 in morphogenesis. Sec14 has been proposed to regulate the DAG pool necessary for proper secretory function (Kearns *et al.*, 1997). Therefore, PLD1 might contribute to morphogenesis by ensuring that adequate levels of DAG are available for the secretion of components to the growing hyphal cell.

There are a number of possible important or even essential functions for PLD1. Nevertheless, disruption of the corresponding gene had only a minor effect. This may be due to a second PLD activity. However, our data suggest that a second PLD gene does exist or is expressed at very low levels. The fact that *PLD1* is not an essential gene suggests that the cell is able to adapt to the loss of PLD1 activity by producing the necessary metabolites in different, PLD1-independent, ways. Since DAG is essential for viability, it is reasonable to propose that adequate levels of DAG can be produced by alternative pathways such as a phospholipase C. In fact, a gene encoding a phosphatidylinositol-specific phospholipase C (*CAPLC1*) has recently been cloned in *C. albicans* (Bennett *et al.*, 1998). In addition to phospholipase C1-derived DAG, DAG can be generated by the action of inositol-phosphoryl ceramide synthase (encoded by *AUR1*) which transfers phosphoinositol from phosphatidylinositol to phytoceramide with the release of DAG (Hashida-Okado *et al.*, 1996). Another potential source for compensatory increases in DAG levels is dephosphorylation of PA generated by acylation of glycerol 3-phosphate (Athenstaedt & Daum, 1997) instead of by hydrolysis of PC. Thus the cell has numerous mechanisms by which to generate DAG and PA, the two prominent products of PLD1 activity.

It has been reported recently that *C. albicans* may be capable of mating (Magee & Magee, 2000; Hull *et al.*, 2000). Such a capability would suggest that this organism is also capable of undergoing sporulation and meiosis. In fact, homologues of several *S. cerevisiae* genes involved in sporulation and meiosis have been identified. PLD1 is critical to the successful completion of meiosis and sporulation in *S. cerevisiae*. It will be interesting to determine whether homologues of sporulation regulators are able to alter the activity of PLD1 in *C. albicans* and whether *PLD1* may be involved in sporulation of *C. albicans*.

The importance of lipid signalling in the metabolism

and pathogenesis of fungi is only now becoming apparent. While the available information is very incomplete, it is already clear that lipid signalling has the potential to be as important to the cell as protein kinase signalling. Also of significance will be the manner in which cells respond to mutations that impact on the ability to utilize lipid messengers. Finally, the manner in which the cells are able to integrate the complex intracellular signals derived from so many different pathways and stimuli into a single coherent response represents a very rich area for future study.

ACKNOWLEDGEMENTS

We thank Dr S. Scherer, University of Minnesota, USA, for providing the fosmid library; Dr W. A. Fonzi, University of Washington, USA, for strains CAI4 and CAF2-1; Dr J. Ernst, University of Düsseldorf, Germany, for the *efg1* mutant; Dr G. Fink, Massachusetts Institute of Technology, USA for the *cph1* and *efg1/cph1* mutants; Dr J. Gerst, Weizman Institute of Science, Israel, for the *SPO14* gene; Dr E. Balish, Medical University of South Carolina, USA, for the mice data, and M. L. Hairfield, Medical University of South Carolina, USA, for the analysis of PLD1 activity in our mutants. This work was supported by the Deutsche Forschungsgemeinschaft (Grant Hu528/7-1) to B.H. and by MUSC Institutional Funds for Research to J. W.D.

REFERENCES

- Athenstaedt, K. & Daum, G. (1997). Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 7611–7616.
- Bennett, D. E., McCreary, C. E. & Coleman, D. C. (1998). Genetic characterization of a phospholipase C gene from *Candida albicans*: presence of homologous sequences in *Candida* species other than *Candida albicans*. *Microbiology* **144**, 55–72.
- Braun, B. R. & Johnson, A. D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* **277**, 105–109.
- Brown, A. J. & Gow, N. A. (1999). Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol* **7**, 333–338.
- Buckley, H. R., Price, M. R. & Daneo-Moore, L. (1982). Isolation of a variant of *Candida albicans*. *Infect Immun* **37**, 1209–1217.
- Buffo, J., Herman, M. A. & Soll, D. R. (1984). A characterization of pH-regulated dimorphism in *Candida albicans*. *Mycopathologia* **85**, 21–30.
- Colthurst, D. R., Schauder, B. S., Hayes, M. V. & Tuite, M. F. (1992). Elongation factor 3 (EF-3) from *Candida albicans* shows both structural and functional similarity to EF-3 from *Saccharomyces cerevisiae*. *Mol Microbiol* **6**, 1025–1033.
- Cook, J. G., Bardwell, L., Kron, S. J. & Thorner, J. (1996). Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast *Saccharomyces cerevisiae*. *Genes Dev* **10**, 2831–2848.
- Ella, K. M., Dolan, J. W. & Meier, K. E. (1995). Characterization of a regulated form of phospholipase D in the yeast *Saccharomyces cerevisiae*. *Biochem J* **307**, 799–805.
- Ella, K. M., Dolan, J. W., Qi, C. & Meier, K. E. (1996). Characterization of *Saccharomyces cerevisiae* deficient in expression of phospholipase D. *Biochem J* **314**, 15–19.
- Ernst, J. (2000). Dimorphism in *Candida albicans*. In *Contributions to Microbiology: Dimorphism in Human Pathogenic and Apathogenic Yeasts*, pp. 98–111. Edited by J. F. Ernst & A. Schmidt. Basel: Karger.
- Feng, Q., Summers, E., Guo, B. & Fink, G. (1999). Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J Bacteriol* **181**, 6339–6346.
- Fodstad, Ø., Hansen, C. T., Cannon, G. B. & Boyd, M. R. (1984). Immune characteristics of the beige-nude mouse: a model for studying immune surveillance. *Scand J Immunol* **20**, 267–272.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.
- Gadd, G. M. (1995). Signal transduction in fungi. In *The Growing Fungus*. Edited by N. A. R. Gow & G. M. Gadd. London: Chapman & Hall.
- Gadd, G. M. & Foster, S. A. (1997). Metabolism of inositol 1,4,5-trisphosphate in *Candida albicans*: significance as a precursor of inositol polyphosphates and in signal transduction during the dimorphic transition from yeast cells to germ tubes. *Microbiology* **143**, 437–448.
- Gaits, F., Fourcade, O., Le Balle, F. & 8 other authors (1997). Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. *FEBS Lett* **410**, 54–58.
- Gillum, A. M., Tsay, E. Y. H. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* **198**, 179–182.
- Gow, N. A. & Gooday, G. W. (1982). Vacuolation, branch production and linear growth of germ tubes in *Candida albicans*. *J Gen Microbiol* **128**, 2195–2198.
- Gow, N. A. R., Robbins, P. W., Brown, A. J. P., Fonzi, W. A., Chapman, T. & Kinsman, O. K. (1994). A hyphal-specific chitin synthase gene (*CHS2*) is not essential for growth, dimorphism or virulence of *Candida albicans*. *Proc Natl Acad Sci USA* **91**, 6216–6220.
- Goyal, S. & Khuller, G. K. (1992). Phospholipid composition and subcellular distribution in yeast and mycelial forms of *Candida albicans*. *J Med Vet Mycol* **30**, 355–362.
- Hashida-Okado, T., Ogawa, A., Endo, M., Yasumoto, R., Takesako, K. & Kato, I. (1996). *AUR1*, a novel gene conferring aureobasidin resistance on *Saccharomyces cerevisiae*: a study of defective morphologies in Aur1p-depleted cells. *Mol Gen Genet* **251**, 236–244.
- Hube, B., Monod, M., Schofield, D. A., Brown, A. J. P. & Gow, N. A. R. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* **14**, 87–99.
- Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schafer, W., Brown, A. J. & Gow, N. A. (1997). Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. *Infect Immun* **65**, 3529–3538.
- Hube, B., Stehr, F., Bossenz, M., Mazur, A., Kretschmar, M. & Schäfer, W. (2000). Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Arch Microbiol* **174**, 362–367.
- Hull, C. M., Raisner, R. M. & Johnson, A. D. (2000). Evidence for mating of the 'asexual' yeast *Candida albicans* in a mammalian host. *Science* **289**, 307–310.
- Kanoh, H., Nakashima, S., Zhao, Y., Sugiyama, Y., Kitajima, Y. & Nozawa, Y. (1998). Molecular cloning of a gene encoding phospholipase D from the pathogenic and dimorphic fungus *Candida albicans*. *Biochim Biophys Acta* **1398**, 359–364.

- Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S. & Bankaitis, V. A. (1997). Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature* **387**, 101–105.
- Lemmon, M. A., Ferguson, K. M. & Schlessinger, J. (1996). PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85**, 621–624.
- Li, X., Routt, S. M., Xie, Z., Cui, X., Fang, M., Kearns, M. A., Bard, M., Kirsch, D. R. & Bankaitis, V. A. (2000). Identification of a novel family of nonclassic yeast phosphatidylinositol transfer proteins whose function modulates phospholipase D activity and Sec14p-independent cell growth. *Mol Biol Cell* **11**, 1989–2005.
- Liu, H., Kohler, J. & Fink, G. R. (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. *Science* **266**, 1723–1726.
- Lopez, M. C., Nicaud, J. M., Skinner, H. B., Vergnolle, C., Kader, J. C., Bankaitis, V. A. & Gaillardin, C. (1994). A phosphatidylinositol/phosphatidylcholine transfer protein is required for differentiation of the dimorphic yeast *Yarrowia lipolytica* from the yeast to the mycelial form. *J Cell Biol* **125**, 113–127.
- McLain, N. & Dolan, J. W. (1997). Phospholipase D activity is required for dimorphic transition in *Candida albicans*. *Microbiology* **143**, 3521–3526.
- Magee, B. B. & Magee, P. T. (2000). Induction of mating in *Candida albicans* by construction of MTL α and MTL β strains. *Science* **289**, 310–313.
- Magee, P. & Scherer, S. (1998). Genome mapping and gene discovery in *Candida albicans*. *ASM News* **64**, 505–511.
- Mago, N. & Khuller, G. K. (1990). Subcellular localization of enzymes of phospholipid metabolism in *Candida albicans*. *J Med Vet Mycol* **28**, 355–362.
- Maneu, V., Cervera, A. M., Martinez, J. P. & Gozalbo, D. (1996). Molecular cloning and characterization of a *Candida albicans* gene (EFB1) coding for the elongation factor EF-1 β . *FEMS Microbiol Lett* **145**, 157–162.
- Mattia, E., Carruba, G., Angiolella, L. & Cassone, A. (1982). Induction of germ tube formation by *N*-acetyl-D-glucosamine in *Candida albicans*: uptake of inducer and germinative response. *J Bacteriol* **152**, 555–562.
- Mayr, J. A., Kohlwein, S. D. & Paltauf, F. (1996). Identification of a novel, Ca(2+)-dependent phospholipase D with preference for phosphatidylserine and phosphatidylethanolamine in *Saccharomyces cerevisiae*. *FEBS Lett* **393**, 236–240.
- Monteoliva, L., Sanchez, M., Pla, J., Gil, C. & Nombela, C. (1996). Cloning of *Candida albicans* sec14 gene homologue coding for a putative essential function. *Yeast* **12**, 1097–1105.
- Moolenaar, W. H. (1995). Lysophosphatidic acid, a multi-functional phospholipid messenger. *J Biol Chem* **270**, 12949–12952.
- Morris, A. J., Frohman, M. A. & Engebrecht, J. (1997). Measurement of phospholipase D activity. *Anal Biochem* **252**, 1–9.
- Riggle, P. J., Slobodkin, I. V., Brown, D. H., Jr, Hanson, M. P., Volkert, T. L. & Kumamoto, C. A. (1997). Two transcripts, differing at their 3' ends, are produced from the *Candida albicans* SEC14 gene. *Microbiology* **143**, 3527–3535.
- Roberts, R. L. & Fink, G. R. (1994). Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev* **8**, 2974–2985.
- Rose, K., Rudge, S. A., Frohman, M. A., Morris, A. J. & Engebrecht, J. (1995). Phospholipase D signaling is essential for meiosis. *Proc Natl Acad Sci USA* **92**, 12151–12155.
- Rudge, S. A., Morris, A. J. & Engebrecht, J. (1998). Relocalization of phospholipase D activity mediates membrane formation during meiosis. *J Cell Biol* **140**, 81–90.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schaller, M., Korting, H. C., Schäfer, W. & Hube, B. (1998). Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microbiol* **29**, 605–615.
- Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A. V., Witke, W., Huttner, W. B. & Soling, H. D. (1999). Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature* **401**, 133–141.
- Sreenivas, A., Patton-Vogt, J. L., Bruno, V., Griac, P. & Henry, S. A. (1998). A role for phospholipase D (Pld1p) in growth, secretion, and regulation of membrane lipid synthesis in yeast. *J Biol Chem* **273**, 16635–16638.
- Stoldt, V. R., Sonneborn, A., Leuker, C. E. & Ernst, J. F. (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J* **16**, 1982–1991.
- Tall, E., Dorman, G., Garcia, P. & 8 other authors (1997). Phosphoinositide binding specificity among phospholipase C isozymes as determined by photo-cross-linking to novel substrate and product analogs. *Biochemistry* **36**, 7239–7248.
- Vasquez-Torres, A., Jones-Carson, J., Wagner, R. D., Warner, T. & Balish, E. (1999). Early resistance of interleukin-10 knockout mice to acute systemic candidiasis. *Infect Immun* **67**, 670–674.
- Waksman, M., Eli, Y., Liscovitch, M. & Gerst, J. E. (1996). Identification and characterization of a gene encoding phospholipase D activity in yeast. *J Biol Chem* **271**, 2361–2364.
- Waksman, M., Tang, X., Eli, Y., Gerst, J. E. & Liscovitch, M. (1997). Identification of a novel Ca²⁺-dependent, phosphatidylethanolamine-hydrolyzing phospholipase D in yeast bearing a disruption in PLD1. *J Biol Chem* **272**, 36–39.
- Wang, B., Biron, C., She, J., Higgins, K., Sunshine, M. J., Lacy, E., Lonberg, N. & Terhorst, C. (1994). A block in both early T lymphocyte and natural killer cell development in transgenic mice with high-copy numbers of the human CD3E gene. *Proc Natl Acad Sci USA* **91**, 9402–9406.
- Xie, Z., Fang, M., Rivas, M. P., Faulkner, A. J., Sternweis, P. C., Engebrecht, J. A. & Bankaitis, V. A. (1998). Phospholipase D activity is required for suppression of yeast phosphatidylinositol transfer protein defects. *Proc Natl Acad Sci USA* **95**, 12346–12351.

Received 13 June 2000; revised 27 November 2000; accepted 22 December 2000.