Functional analysis of the phospholipase C gene
CaPLC1 and two unusual phospholipase C genes,
CaPLC2 and CaPLC3, of Candida albicans

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Phospholipases C are known to be important regulators of cellular processes but may also act as virulence factors of pathogenic microbes. At least three genes in the genome of the human-pathogenic fungus Candida albicans encode phospholipases with conserved phospholipase C (Plc) motifs. None of the deduced protein sequences contain N-terminal signal peptides, suggesting that these phospholipases are not secreted. In contrast to its orthologue in Saccharomyces cerevisiae, CaPLC1 seems to be an essential gene. However, a conditional mutant with reduced transcript levels of CaPLC1 had phenotypes similar to Plc1p-deficient mutants in S. cerevisiae, including reduced growth on media causing increased osmotic stress, on media with a non-glucose carbon source, or at elevated or lower temperatures, suggesting that CaPlc1p, like the Plc1p counterpart in S. cerevisiae, may be involved in multiple cellular processes. Furthermore, phenotypic screening of the heterozygous Δcaplc1/ CaPLC1 mutant showed additional defects in hyphal formation. The loss of CaPLC1 cannot be compensated by two additional PLC genes of C. albicans (CaPLC2 and CaPLC3) encoding two almost identical phospholipases C with no counterpart in S. cerevisiae but containing structural elements found in bacterial phospholipases C. Although the promoter sequences of CaPLC2 and CaPLC3 differed dramatically, the transcriptional pattern of both genes was similar. In contrast to CaPLC1, CaPLC2 and CaPLC3 are not essential. Although Caplc2/3 mutants had reduced abilities to produce hyphae on solid media, these mutants were as virulent as the wild-type in a model of systemic infection. These data suggest that C. albicans contains two different classes of phospholipases C which are involved in cellular processes but which have no specific functions in pathogenicity.

INTRODUCTION

Induction of phosphoinositide (PI)-specific phospholipase C (PI-Plc) causes hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], thereby generating two intracellular second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). DAG activates a family of phospholipid-dependent protein kinases (Nishizuka, 1992). IP3 stimulates release of Ca2+ ions that activate calmodulin-dependent enzymes (Berridge, 1993). Furthermore, IP3 can also be a precursor of inositol polyphosphates such as IP5 or IP6, whose signalling functions have not been fully elucidated (York et al., 1999). Therefore, phospholipases C are important regulators in many signal transduction pathways.

Abbreviations: DAG, 1,2-diacylglycerol; IP3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PI, phosphoinositide; PI-Plc, PI-specific phospholipase C.
Mammalian PI-Plcs are grouped into four subfamilies (β, γ, δ and ε) whose structural organization shares an X and a Y domain, but differs in elements necessary for their specific functions and localizations (Rhee, 2001). In lower eukaryotes, such as yeasts, only δ isoforms have been found, leading to the hypothesis that this isoform represents an `archetypal phospholipase C' (Ochocka & Pawelczyk, 2003; Rhee, 2001). Similarities between mammalian and bacterial PI-Plcs are reduced to a few residues within the N-terminal part, representing the X domain (Griffith & Ryan, 1999).

A gene encoding a PI-Plc (PLC1) has been identified in *Saccharomyces cerevisiae* (Flick & Thorner, 1993; Payne & Fitzgerald-Hayes, 1993; Yoko-o et al., 1993) and depending on the genetic background, PLC1 in *S. cerevisiae* can be essential (Yoko-o et al., 1993). In other studies, disruption of PLC1 revealed a large number of phenotypic responses. For example, plc1 mutants showed osmosensitivity at high concentrations of carbohydrates or NaCl, chromosome missegregation, temperature sensitivity and reduced growth rates in media containing non-glucose carbon sources (Flick & Thorner, 1993; Payne & Fitzgerald-Hayes, 1993). Some of these phenotypes (chromosome missegregation, temperature sensitivity) could be partially restored with exogenous Ca²⁺ ions, which supported the view that PLC1 is involved in Ca²⁺-signal transduction of *S. cerevisiae*. PLC1 was shown to be essential for glucose-stimulated PI turnover and subsequent activation of plasma-membrane H⁺-ATPases (Coccetti et al., 1998), and mutants lacking PLC1 failed to regulate intracellular Ca²⁺ concentrations in response to extracellular glucose, suggesting a role for Plc1p in transducing glucose signals (Tisi et al., 2002). Furthermore, Ansari et al. (1999) showed that Plc1p interacts with the receptor-like protein Gpr1p as a component of the nitrogen-signalling pathway for formation of pseudohyphae in *S. cerevisiae*. Based on these results it was suggested that Plc1p acts in two filamentation pathways, the cAMP- and the Ras2-controlled (mitogen-activated protein kinase, MAPK) pathway.

While traditionally, Plc activity has been thought to be associated with the plasma membrane, a putative nuclear localization sequence was described in an early report of PLC1 (Payne & Fitzgerald-Hayes, 1993), and York et al. (1999) and Odom et al. (2000) demonstrated a nuclear function of Plc1p within an inositol polyphosphate pathway for mRNA export and regulation of the transcriptional complex ArgR-Mcm1 (reviewed by York et al., 2001). Moreover, Plc1p physically interacts with the nuclear protein Sgd1p, with Ndc10 and Cep3 (two proteins of the kinetochore complex), and with Tor2, a protein involved in actin structure formation (Lin et al., 1998, 2000, 2002). These results show that Plc1p acts as an important multifunctional protein at several locations within the yeast cell.

*Candida albicans* is a human-pathogenic fungus of increasing clinical importance closely related to *S. cerevisiae*. Among the most prominent virulence factors of *C. albicans* are extracellular hydrolytic enzymes and the ability to grow either in a yeast or a hyphal form (dimorphism) (Calderone & Fonzi, 2001). Extracellular hydrolases include proteinases (Naglik et al., 2004), lipases (Hube et al., 2000) and phospholipases (Ghannoum, 2000), the latter with phospholipase A and B activities (Ghannoum, 2000). The dimorphic transition is regulated by a number of signal transduction pathways such as the MAPK and cAMP/protein kinase A pathways (reviewed by Sudbery et al., 2004; Whiteway & Oberholzer, 2004). In addition, lipid signalling pathways with second messengers such as DAG may play a role during morphogenesis of *C. albicans* (Hube et al., 2001).

Interestingly, while PLC1 is the only known gene encoding a PI-Plc in *S. cerevisiae* (Jun et al., 2004), at least two PLC genes have been identified in *C. albicans*. The first PLC gene identified in *C. albicans* (named CAPLC1) is an orthologue of PLC1 of *S. cerevisiae* (Bennett et al., 1998). CAPLC1, which has been shown to be transcribed in both the yeast and hyphal forms of *C. albicans*, encodes a large protein of 1099 amino acids with classical X and Y domains (27% homology to ScPlc1p), with phospholipase C activity when expressed in *Escherichia coli*. The protein encoded by the second gene (named PIPLC) is more similar to extracellular bacterial phospholipases C than to the phospholipases C from higher eukaryotes (Andaluz et al., 2001). PIPLC was described as a significantly smaller gene compared to CAPLC1 (1029 bp), has a presumptive homologue in the neighbourhood of the gene LIG4 and produced DAG when expressed in *E. coli*. The fact that this gene has no counterpart in *S. cerevisiae* may suggest that it evolved during adaptation of *C. albicans* to the human host. However, the function of PLC genes in *C. albicans* is unknown.

The aim of this project was to study the function of these two different types of PI-PLC genes in *C. albicans*. Therefore, we have investigated the role of CAPLC1 (named CaPLC1 in this study), PIPLC (renamed CaPLC2 in this study) and a third PLC gene (named CaPLC3) in growth, cellular functions, morphology and virulence.

**METHODS**

**Strains and growth conditions.** We used *C. albicans* strains SC5314 and CAF2-1 as wild-type controls and CAH4 to produce mutants either lacking or overexpressing phospholipase genes (Fonzi & Irwin, 1993). Cells were grown on YPD [2 % (w/v) glucose, 1 % (w/v) yeast extract, 2 % (w/v) peptone] or SD [2 % (w/v) glucose, 0-5 % (w/v) (NH₄)₂SO₄, 0-17 % (w/v) yeast nitrogen base without amino acids and ammonium sulfate; Difco] solid media prior to use. For phenotype screening the following media were used: M199 (Sigma), CAA medium (Leuker et al., 1997; Stoldt et al., 1997), Sabouraud glucose medium [4 % (w/v) glucose, 1 % (w/v) peptone], YCB-BSA (Hube et al., 1994), blood agar [11 bouillon 0-3 % (w/v) NaCl, 0-3 % (w/v) Na₂HPO₄, 2H₂O, 1 % (w/v) peptone, 1-4 % (w/v) Lab-lemco powder (Oxoid), 15 g agar, 50 ml mutton blood], Spider medium (Liu et al., 1994), Lee’s medium pH 4-5 and 6-5 (Buffo et al., 1984), and egg yolk agar (Fu et al., 1997).

For studying the PI-Plc inhibitor 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphorylcholine (ET-18), hyphal formation was induced by pH- and temperature-shift (Buffo et al., 1984) and inhibitor was
added at concentrations of 10, 20 and 200 μM. Three independent samples were taken after 1, 2, 3, 4 and 5 h to determine the percentages of hyphal formation. For phenotypic screening of mutant strains a serial drop dilution test was used. Samples (5 μl) of suspensions with different concentrations of cells were dropped onto the corresponding solid media. For screening the conditional mutant ΔCaplc1/pMET3-CaPLC1, wild-type and mutant cells were pre-cultured in SD supplemented with 2.5 mM methionine and 2.5 mM cysteine at 37 °C for 1 day prior to counting. To analyse growth of the heterozygous mutant Caplc1/CaPLC1 in non-glucose media, cells were incubated in SD without glucose, but with 3 % (v/v) glycerol or 2 % (w/v) potassium acetate. For expression studies the following growth conditions were used. For expression of CaPLC1 and genes possibly associated with CaPLC1 functions, CA2-1 was grown overnight at 30 °C in SD medium, diluted into fresh SD medium to give a concentration of 10^6 cells ml^-1 and incubated at 37 °C to an OD600 of 0.6. The culture was then divided into 10 ml aliquots, centrifuged and the pellet resuspended in 40 ml of the following media: SD at 37 °C, SD at 42 °C (heat shock), SD containing 60 μM cadmium at 37 °C and SGlyc [SD with 2 % (v/v) glycerol instead of 2 % (w/v) glucose] at 37 °C. After 1 h incubation, cells were harvested for RNA extraction (see below). For expression of PLC2 and PLC3, an overnight culture of SC5314 was grown in SD at 30 °C, diluted into SD (1:100), YPD (1:100), or Sabouraud glucose (SG) (1:1000) and incubated at 37 °C for 1 h. For the cassette were amplified using the primers PLC1-3 and PLC1-4 (Table 1) as template. This PCR fragment was ligated into the downstream region of the hisG-URA3-hisG cassette of pMB7 and named pDB104. pDB104 was linearized with PvuI prior to transformation and used to disrupt the first allele of CaPLC1.

Construction of pDB104. A 716 bp PCR fragment containing the region between 1043 bp and 1759 bp of CaPLC1 (accession no. Y13975), was cloned into pMB7 (Fonzi & Irwin, 1993) upstream of the hisG-URA3-hisG cassette. A second PCR fragment representing 2668 bp and 3445 bp of CaPLC1 was ligated into the downstream region of the hisG-URA3-hisG cassette of pMB7 and named pDB104. pDB104 was linearized with PvuI prior to transformation and used to disrupt the first allele of CaPLC1.

Construction of pDK-4. pDK-4 contains an allele-specific CaPLC1-disruption cassette. PCR products for the gene-specific regions of the cassette were amplified using the primers PLC1-3 and PLC1-4 (Table 1) with DNA from the heterozygous ΔCaplc1/CaPLC1 mutant as template. This PCR fragment was ligated into pCR2.1-TOPO (Invitrogen) to produce pDK-1. The fragment was subcloned into pGEM-T Easy (Promega) to give pDK-2. The selection marker URA3 was amplified from pMB7 with primers URA1-1 and URA2-1 and ligated into pCR2.1-TOPO (pDK-3). Finally, the URA3 fragment of pDK-3 was cloned into the CaPLC1 fragment within pDK-2 to give pDK-4. pDK-4 was linearized prior to transformation of the Ura^- heterozygous ΔCaplc1/CaPLC1 mutant.

Construction of pDS952. A 4299 bp PCR fragment containing bp 8921 to bp 14120 of contig 6-2335 flanked by XbaI and XhoI sites was cloned into pBluescript. A second PCR fragment was produced by inverse PCR. The hisG-URA3-hisG cassette of pMB7 was cloned into this fragment, leaving 501 bp and 495 bp flanking regions of CaPLC1 for homologous recombination.

### Table 1. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tr>
<td>PLC1-1</td>
<td>ATG TTG GAA TCA TTA AAT GGT C</td>
</tr>
<tr>
<td>PLC1-2</td>
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<tr>
<td>PLC1-3</td>
<td>TTT GGG ATT CAT TAG CTC AAG G</td>
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<tr>
<td>PLC1-4</td>
<td>TTG TAT CGA CAT TGC TAC TGC</td>
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</tr>
<tr>
<td>PLC2-0</td>
<td>CAA TCG ATT GGT TTC TAT TTC AAT</td>
</tr>
<tr>
<td>PLC2-1</td>
<td>CAA AAC TTG GGT AAA GGA TAT TGA</td>
</tr>
<tr>
<td>PLC2-2</td>
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</tr>
<tr>
<td>PLC2-4</td>
<td>TTA GTA TCA ATT AAA GCT TCA GGC</td>
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<td>PLC2-6</td>
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<td>GAT TAG AAG AGT TGC TAA GTA</td>
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</tr>
<tr>
<td>PLC1-9</td>
<td>TTG TAT CGA CAT TGC TAC TGC</td>
</tr>
</tbody>
</table>

For cloning procedures E. coli TOP 10 cells (Invitrogen) were used as described by the manufacturer.

### Disruption of CaPLC1

Four different disruption cassettes were produced to disrupt CaPLC1, as follows.

### Construction of cascade 4 (oligomer-based). We also aimed to disrupt CaPLC1 with a PCR product that had homologous oligonucleotide sequences to the CaPLC1 gene sequence on each end of the
lysing a number of restriction digests of pDK-16 and pDK-18.

Table 1. This disruption cassette was amplified with the plasmid pGEM-URA3 as template.

Construction of a conditional CaPLC1 mutant. In order to produce a conditional CaPLC1 mutant we used primers PLC1-7 and PLC1-8 flanked by a BamHI and a PstI site to amplify a 1350 bp PCR product containing a fragment of the 5′ end of CaPLC1 beginning with the ATG start codon. This fragment was cloned into the BamHI/PstI sites of pCaDis (Care et al., 1999) to give pLM14. pLM14 was linearized with CiaI and transformed into the Ura− heterozygous Δcaplci/CaPLC1 mutant. Integration into the non-disrupted allele was confirmed by Southern analysis and PCR using primers pMET1 and PLC1-9. Integration into the non-disrupted allele was shown by the amplification of a 2.8 kb PCR product containing a HindIII site, while integration into the disrupted allele resulted in an amplicon of 3 kb without a HindIII site.

Cloning and sequencing of CaPLC2 and CaPLC3. The genes were amplified using the Expand Long Template PCR System (Roche), genomic DNA of SC5314 and the primers PLC2-9, PLC2-10, PLC2-3 and PLC3-3. PCR products were ligated into pGEM-T Easy and transformed into E. coli TOP 10 (Invitrogen). PCR fragments were sequenced using T7 or gene-specific primers (PLC2-0/PLC2-10 and PLC3-2/PLC3-3) (Table 1). Sequences were compared with sequences of PI-PLC (AJ277538) (Andaluz et al., 2001) and the Candida database (http://www.Pasteur.fr/recherche/unites/Galar-Fungail) of the Institut Pasteur, France, using the BLASTN search mode.

Disruption of CaPLC2 and CaPLC3. Using primers PLC2-5 and PLC2-6 almost the complete ORF of CaPLC2 was amplified using genomic DNA of SC5314, and the corresponding PCR product was ligated into pGEM-T Easy, generating pDK-6. The fragment was sequenced and the sequence compared with published data. A HindIII restriction site downstream of the insert was deleted by linearizing the plasmid with HindIII, treatment with mung bean nuclease (MBI Fermentas) followed by religation, to generate pDK-7. The deletion was confirmed by sequencing. Primers PLC2-7 and PLC2-8 were then used to amplify a PCR fragment that contained pDK-7 except for 135 bp within the ORF by inverse PCR. The fragment was ligated with the hisG-URA3-hisG cassette to give pDK-8. pDK-8 was linearized prior to transformation into C. albicans. Due to the high similarity of CaPLC2 and PLC3, pDK-8 was used for the deletion of both genes.

Overexpression of CaPLC2 and CaPLC3. In order to rescue the wild-type phenotype in mutants lacking CaPLC2 and CaPLC3, and to overexpress CaPLC2 or CaPLC3, both genes were cloned behind the PCK1 promoter of plasmid pBl-1 (Leuker et al., 1997; Stoldt et al., 1997). CaPLC2 and CaPLC3 were amplified using the primers PLC3-2 and PLC2-10, and PLC3-2 and PLC3-3, respectively, with SC5314 genomic DNA as template and cloned into the BgllI site of pBl-1, generating pDK-16 and pDK-18. Integration of CaPLC2 and CaPLC3 downstream of the PCK1 promoter was confirmed by analyzing a number of restriction digests of pDK-16 and pDK-18.

Transformation of C. albicans. Two modified lithium acetate protocols (Sanglard et al., 1996) and one electroporation protocol (De Backer et al., 1999) were used. Transformants were screened using PCR and Southern blot analysis as described previously (Feld et al., 2002).

RNA extraction and RT-PCR. Liquid cultures were grown as described for each experiment, cells harvested and RNA isolated as described previously (Feld et al., 2002). For RT-PCR, 1-5 μg RNA was DNase treated and cDNA synthesized (Feld et al., 2002). Controls included cDNA synthesis without addition of reverse transcriptase and amplification of an intron-containing fragment of the EFB1 gene (Schaller et al., 1999) (with primers EFB1-1 and EFB1-2, Table 1). To ensure that samples from the exponential phase of PCR amplification were examined, we used 20, 25, 30, 35 and 40 cycles, respectively, for genes of interest. All RT-PCR experiments were done at least in duplicate with samples from two independent biological experiments. The housekeeping gene ACT1, which was expressed to a similar extent under all conditions investigated, was used as an additional internal standard control.

Microarray hybridization and analysis. For transcript profiling, we used C. albicans microarrays (Eurogentec) containing 6039 ORFs. Arrays were designed as described under http://www.pasteur.fr/recherche/unites/Galar-Fungailarrays.html. Information about coding sequences and proteins was obtained from the CandidaDB database (http://genolist.pasteur.fr/CandidaDB/). RNA was reverse transcribed into cDNA and labelled with Cy3 or Cy5 (dye swap) (Sigle et al., 2005). Labelled cDNA was hybridized to the C. albicans arrays as described by Sigle et al. (2005). Hybridized slides were scanned with an Axon 4000B scanner at a 10 μm resolution. Data were extracted by GenePix 4.1 software (Axon). An intensity-dependent data normalization (LOWESS) was performed in GeneSpring 6.0. The different sets of data were compared to each other by a one-way analysis of variance (ANOVA) test with a P-value cut-off of 0.099 for genes that had an intensity in both channels higher than 100. Each gene that passed this test and showed at least 1.5-fold change in two arrays was defined as differentially expressed.

Systemic mouse infection. C. albicans strains (Δplc2/Δplc3, CAF2-1) were used in a mouse model of systemic infection as described previously (Fradin et al., 2005).

RESULTS

Disruption of CaPLC1 in C. albicans

CaPLC1 is a 2997 bp gene encoding a protein with PI-specific phospholipase C activity and a predicted molecular mass of 124.6 kDa (Bennett et al., 1998). The gene is localized on chromosome 7 between HIS7, possibly involved in histidine biosynthesis, and ENP1, annotated as an essential nuclear protein by homology (http://www.pasteur.fr/recherche/unites/Galar-Fungail/). Sequence analysis of the deduced protein revealed no hints of an N-terminal signal peptide or any other sequence that may target the protein to the secretory pathway or mitochondria, suggesting that the protein is localized in the cytoplasm. However, the frequent appearance of basic amino acids (Lys, Arg) between the X and Y domains of CaPlic1p (amino acids 718–790) points to a nuclear localization (Dingwall & Laskey, 1991).

In order to investigate the function of CaPlic1p we prepared a disruption cassette to delete CaPLC1 in a wild-type background (strain CAI4). Plasmid pDB104 was used to delete an internal 900 bp fragment, containing a HindIII site, of CaPLC1. After a first round of transformation with pDB104, a number of clones isolated had this fragment deleted in one allele of CaPLC1 as shown by PCR and Southern analysis. However, even after screening more than 190 clones following a second round of transformation with
pDB104, no clone was identified in which both alleles of CaPLC1 were deleted. Since it has been shown that allele-specific gene targeting in C. albicans may result from heterozygosity between alleles (Yeast & Fonzi, 2000), we constructed an allele-specific cassette with gene-specific sequences that were deleted in the heterozygous mutant. Therefore, integration by homologous recombination should only be possible into the second non-disrupted allele. Furthermore, we used a third cassette with flanking sequences representing non-translated regions of CaPLC1 and a fourth cassette based on allele-specific flanking oligonucleotides (Wilson et al., 1999). None of the transformants obtained with these disruption cassettes had all alleles disrupted. In total, an additional 500 clones obtained by different transformation protocols using these cassettes in more than 28 transformation experiments were screened. Although the vast majority of clones showed integration into the first allele (non-allele-specific cassettes) or random integration into the genome (allele-specific cassettes), we identified seven clones where integration into the second allele occurred. However, in these clones, additional DNA fragments were identified which contained the non-disrupted CaPLC1 allele, suggesting duplication of these regions prior to integration of the cassettes into the corresponding region. These data strongly suggest that CaPLC1 is an essential gene.

Construction of a conditional CaPLC1 mutant

Since all attempts to generate a CaPLC1 null mutant failed and disruption of the second allele was only possible in mutants where the chromosomal CaPLC1 region had been duplicated, we decided to produce a conditional mutant. Since the MET3 promoter of C. albicans (pMET3), first analysed by Care et al. (1999), was shown to be a tightly regulated promoter and appropriate for studying essential genes, we used the plasmid pCaDis (Care et al., 1999) to prepare a CaPLC1 mutant with a disrupted allele and a non-disrupted allele under the control of the MET3 promoter. Addition of 2.5 mM methionine and cysteine was shown to repress the MET3 promoter (Care et al., 1999). However, ΔCaP1/pMET3-CaPLC1 mutants were still viable on media containing methionine and cysteine (2.5 mM each). In order to investigate the transcript levels of CaPLC1 in these mutants under conditions that repress the MET3 promoter, we used RT-PCR and CaPLC1-specific primers. Although expression levels of CaPLC1 were significantly reduced in media containing methionine and cysteine, transcripts were still detectable (not shown). Since the level of CaPLC1 transcripts in wild-type strains was very low compared to housekeeping genes (Fig. 1), we reasoned that the low level of transcription of CaPLC1 in the controllable mutant was sufficient for survival. However, since the level of transcription of CaPLC1 in the ΔCaP1/pMET3-CaPLC1 mutant was reduced under repression of the MET3 promoter, compared to CaPLC1 levels in wild-type cells, we used this conditional mutant for a broad phenotype screening.

Phenotypic screening of ΔCaP1/pMET3-CaPLC1

A first phenotypic screening of the ΔCaP1/pMET3-CaPLC1 mutant, under conditions which repress the MET3 promoter, was based on observations that had been made for plc1 mutants of S. cerevisiae (Flick & Thorner, 1993; Payne & Fitzgerald-Hayes, 1993; Yoko-o et al., 1993). Phenotypes found for plc1 mutants of S. cerevisiae included temperature sensitivity, osmosensitivity, and reduced growth with media containing methionine and cysteine. Under these conditions, ΔCaP1/pMET3-CaPLC1 showed strong growth inhibition at elevated temperatures and weak inhibition at low temperatures under conditions that repressed the MET3 promoter as compared to

![Fig. 1. Expression profile of CaPLC1 and selected genes putatively linked to CaPLC1 function under conditions known to be associated with PLC1 function in S. cerevisiae (GLE1, IPK1, IPK2 (ARG82)) using RT-PCR. The ACT1 gene was used as an internal standard to ensure equal loading of cDNA. Samples were analysed after 20, 25, 30, 35 and 40 PCR cycles (see Methods). G, growth in SGlyc medium at 37 °C; Cd, growth in SD supplemented with cadmium at 37 °C; 37, growth in SD at 37 °C; 42, growth in SD at 42 °C. For details see Methods.](http://mic.sgmjournals.org)
non-repressed conditions (Fig. 2). Furthermore, addition of 5 % (w/v) sorbitol or 1 M NaCl to the growth medium (causing increased osmotic stress) (Fig. 2) or growth on media with galactose as the sole source of carbon instead of glucose (not shown) caused moderate attenuation of growth. Since it was shown that phospholipase C activity in \textit{S. cerevisiae} is necessary for the transcriptional control of the ArgR–Mcm1 complex (Odom et al., 2000), we also tested growth of the \textit{Δcaplc1/pMET3-CaPLC1} mutant on media with arginine as a sole source of nitrogen. Under these conditions, growth was again clearly reduced when the promoter was repressed. Finally, addition of the agent nocodazole, which targets cellular microtubules and inhibits chromosome segregation of the fungus, also reduced the ability to grow under repressed conditions.

**Fig. 2.** Attenuated growth of the \textit{Δcaplc1/pMET3-CaPLC1} conditional mutant under conditions that repress the \textit{MET3} promoter. The wild-type strain CAF2-1 and the \textit{Δcaplc1/pMET3-CaPLC1} mutant were grown for 1 day in liquid SD medium supplemented with 2-5 mM methionine and cysteine to repress the \textit{MET3} promoter. Cells were counted, diluted and 5 μl aliquots containing 5 × 10^4 to 5 cells were dropped onto the different media with and without 2-5 mM methionine and cysteine and incubated as indicated. The upper panel of each pair shows the wild-type, and the lower pair the mutant. SGal, SD medium with 2% galactose instead of glucose; ArgD, SD medium with 1 mg ml⁻¹ arginine as a sole source of nitrogen.

**Phenotypic screening of the heterozygous \textit{Δcaplc1/CaPLC1} mutant**

Since we observed growth phenotypes for the \textit{Δcaplc1/pMET3-CaPLC1} mutant under conditions which repressed the \textit{MET3} promoter but which did not completely block transcription of the \textit{CaPLC1} gene, we reasoned that the heterozygous \textit{Δcaplc1/CaPLC1} mutant may also show altered phenotypes due to reduced mRNA levels as a result of a gene dosage effect. It should be noted that hyphal formation is strongly inhibited in media containing 2-5 mM methionine and cysteine (not shown). Consequently, defects in the ability to produce hyphal cells could not be tested in the conditional mutant. Therefore, we tested the \textit{Δcaplc1/CaPLC1} mutant under various growth conditions, including conditions that favour filamentous growth.

In addition to reduced growth at elevated temperature and in the presence of nocodazole, the heterozygous mutant showed reduced growth at 30 °C and 37 °C in media supplemented with itraconazole (1 μg ml⁻¹) or hydrogen peroxide (880 μM), and in media with glycerol and acetate as sole carbohydrate sources (not shown). Furthermore, hyphal formation was impaired at 37 °C on blood agar, serum agar or solid Spider medium, as filaments appeared delayed in comparison to the CAF2-1 wild-type strain (Fig. 3). Both the wild-type and the heterozygous mutant produced secreted aspartic proteinases (Saps) that permitted growth on YCB-BSA medium. However, hyphal formation and the white zone around colonies due to acidification and precipitation of BSA were retarded for \textit{Δcaplc1/CaPLC1} colonies (Fig. 3).

**The PI-Plc-specific inhibitor ET-18 blocks growth of \textit{C. albicans}**

Since gene disruption experiments suggested that \textit{CaPLC1} is an essential gene, we analysed the effect of the PI-Plc-specific inhibitor 1-O-octadecyl-2-O-methyl-\textit{rac}-glycero-3-phosphorylcholine (ET-18) on growth of \textit{C. albicans}. Cells were precultured in Lee’s medium pH 4-5, 30 °C, and inoculated into Lee’s medium pH 6-5, 37 °C, supplemented with 10, 20 and 200 μM ET-18 or without addition of inhibitor. Three independent samples were taken after 0, 1, 2, 3, 4 and 5 h to determine the percentages of hyphal formation. Addition of ET-18 caused dose-dependent inhibition of hyphal formation (Table 2). At a concentration of 200 μM, ET-18 caused 80 % inhibition of hyphal formation (Table 2) and at ET-18 concentrations higher than 200 μM (not shown) growth was completely prevented, suggesting that PI-Plcs in \textit{C. albicans} are essential for growth.

**Construction of a conditional \textit{CaPLC1} mutant in a \textit{pld1/pld1} genetic background**

One of the hydrolytic products of phospholipase C activity is DAG, an important intracellular second messenger. Since DAG is also produced by other biochemical pathways such as hydrolytic activity of phospholipase D encoded by the gene \textit{PLD1} (Hube et al., 2001) we questioned whether a...
disrupted one allele of \textit{CaPLC1} in the \textit{pld1/pld1} mutant and replaced the native \textit{CaPLC1} promoter with the \textit{MET3} promoter as described above. In addition to the above-described phenotypes for the \textit{ΔCaplc1/pMET3-CaPLC1} mutant, the conditional \textit{pld1/pld1 ΔCaplc1/pMET3-CaPLC1} mutant showed enhanced osmosensitivity at 1 M NaCl (not shown).

**Transcriptional analysis of \textit{CaPLC1} and genes putatively linked to \textit{CaPLC1} function**

Phenotypic screening of mutants lacking \textit{PLC1} in \textit{S. cerevisiae} (Odom et al., 2000) and \textit{C. albicans} (this study) revealed sensitivity to different environmental stresses. Therefore, we questioned whether \textit{CaPLC1} is regulated in response to such stresses. In \textit{S. cerevisiae} the products of the \textit{PLC1}, \textit{GLE1}, \textit{IPK1} and \textit{IPK2} (ARG82) genes are known to be involved in nuclear mRNA export (York et al., 2001) and may be co-regulated. A mutant lacking \textit{GLE1} in \textit{S. cerevisiae} showed temperature sensitivity similar to mutants lacking \textit{PLC1}. Therefore, we examined the transcript level of \textit{CaPLC1} and the orthologous genes of \textit{GLE1}, \textit{IPK1}, \textit{IPK2} in \textit{C. albicans} wild-type cells (\textit{CaGLE1}, IPF15261, CA2378; \textit{CaIPK1}, IPF10566, CA0280; \textit{CaIPK2}, IPF16498, CA2312) under different stress conditions such as heat shock, glycerol as sole source of carbon and exposure to cadmium (Fig. 1). None of the investigated genes had significantly modified transcript levels in these media, although mRNA levels for \textit{CaPLC1}, \textit{GLE1} and \textit{IPK2} seemed moderately increased at 42 °C and for \textit{CaPLC1} moderately reduced in medium containing cadmium (Fig. 1).

**Genome-wide transcriptional profiling of the conditional \textit{ΔCaplc1/pMET3-CaPLC1} mutant**

PI turnover due to the hydrolytic activity of phospholipase C produces not only DAG, but also IP$_3$, another intracellular second messenger that stimulates release of Ca$^{2+}$, activates calmodulin-dependent enzymes and is a substrate for inositol polyphosphates. Therefore, reduced levels of \textit{CaPLC1} transcripts may have an influence on multiple cellular processes, which in turn could cause changes in the global transcriptional profile of the cell. To analyse the transcriptional profile of the conditional mutant we chose the condition that caused the strongest phenotype in the conditional mutant: elevated growth temperature. CAF2-1 and the conditional mutant were precultured in SD at 37 °C, diluted into SD medium supplemented with 2.5 mM methionine and cysteine at a density of 1 x 10$^7$ cells ml$^{-1}$ and incubated for 3 h at 43 °C. RNA was isolated from both the wild-type and the conditional mutant and used to prepare labelled cDNA.

Forty-five genes were found to be differentially regulated in the conditional mutant as compared with the wild-type strain under repressed conditions. Only four genes (\textit{GDH3}, \textit{MET18}, IPF12399 and \textit{CDC46}) were found to be downregulated and 41 genes upregulated (Table 3).
Table 3. Genes upregulated in the conditional ΔCaplc1/pMET3-CaPLC1 mutant at 43 °C in the presence of 2-5 mM methionine and cysteine as compared with the parental wild-type strain under the same conditions.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Systematic name</th>
<th>Normalized fold upregulated</th>
<th>P-value†</th>
<th>Known or putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFT1</td>
<td>CA5433</td>
<td>1:89</td>
<td>0.099</td>
<td>SNARE-like protein (by homology)</td>
</tr>
<tr>
<td>CTR1</td>
<td>CA1496</td>
<td>1:62</td>
<td>0.099</td>
<td>Copper transport protein</td>
</tr>
<tr>
<td>IPF15013</td>
<td>CA2761</td>
<td>1:51</td>
<td>0.097</td>
<td>Pyruvate decarboxylase regulatory protein (by homology)</td>
</tr>
<tr>
<td>IPF5830</td>
<td>CA4855</td>
<td>1:54</td>
<td>0.095</td>
<td>Unknown function</td>
</tr>
<tr>
<td>NBN1</td>
<td>CA4688</td>
<td>1:72</td>
<td>0.094</td>
<td>Involved in chromatin remodelling and possibly transcription regulation (by homology)</td>
</tr>
<tr>
<td>NRG1</td>
<td>CA5289</td>
<td>1:71</td>
<td>0.093</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>GUT2</td>
<td>CA3566</td>
<td>1:78</td>
<td>0.086</td>
<td>Glycerol-3-phosphate dehydrogenase, mitochondrial (by homology)</td>
</tr>
<tr>
<td>IPF10564</td>
<td>CA3328</td>
<td>1:50</td>
<td>0.086</td>
<td>Oxidoreductase, disulfide-oxidoreductase</td>
</tr>
<tr>
<td>IPF17676</td>
<td>CA0411</td>
<td>1:70</td>
<td>0.083</td>
<td>Similar to S. cerevisiae Cst13p involved in chromosome stability (by homology)</td>
</tr>
<tr>
<td>RTA2</td>
<td>CA3607</td>
<td>2:45</td>
<td>0.077</td>
<td>Phospholipid-translocating ATPase activity</td>
</tr>
<tr>
<td>IPF3092</td>
<td>CA4658</td>
<td>1:54</td>
<td>0.077</td>
<td>Unknown function</td>
</tr>
<tr>
<td>CRH11</td>
<td>CA0375</td>
<td>2:17</td>
<td>0.075</td>
<td>Probable membrane protein (by homology); endopeptidase, hydrolase</td>
</tr>
<tr>
<td>GAP7</td>
<td>CA0757</td>
<td>1:52</td>
<td>0.072</td>
<td>General amino acid permease</td>
</tr>
<tr>
<td>CDR1</td>
<td>CA6066</td>
<td>1:77</td>
<td>0.072</td>
<td>ABC transporter</td>
</tr>
<tr>
<td>MSS4</td>
<td>CA0623</td>
<td>1:90</td>
<td>0.068</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase (by homology)</td>
</tr>
<tr>
<td>IFM3</td>
<td>CA3742</td>
<td>1:98</td>
<td>0.065</td>
<td>2-Hydroxyacid dehydrogenase (by homology), glycerate-formate-dehydrogenase-like</td>
</tr>
<tr>
<td>SCW1</td>
<td>CA0156</td>
<td>1:53</td>
<td>0.061</td>
<td>Glucanase (by homology)</td>
</tr>
<tr>
<td>RBT2</td>
<td>CA3957</td>
<td>1:91</td>
<td>0.059</td>
<td>Repressed by TUP1 protein 2; Rbt2p, ferric reductase (by homology)</td>
</tr>
<tr>
<td>HOL4</td>
<td>CA4204</td>
<td>1:67</td>
<td>0.058</td>
<td>Member of major facilitator superfamily multidrug-resistance protein subfamily 1 (by homology)</td>
</tr>
<tr>
<td>ECM331</td>
<td>CA2181</td>
<td>2:22</td>
<td>0.055</td>
<td>Involved in cell wall biogenesis and architecture (by homology)</td>
</tr>
<tr>
<td>IPF11167</td>
<td>CA0759</td>
<td>1:56</td>
<td>0.053</td>
<td>Unknown function</td>
</tr>
<tr>
<td>FRE7</td>
<td>CA5621</td>
<td>2:42</td>
<td>0.047</td>
<td>Ferric reductase transmembrane component (by homology)</td>
</tr>
<tr>
<td>IPF4999</td>
<td>CA2874</td>
<td>2:23</td>
<td>0.045</td>
<td>DNA-binding, two-component response-regulator</td>
</tr>
<tr>
<td>COX19</td>
<td>CA0768</td>
<td>1:53</td>
<td>0.043</td>
<td>Protein required for cytochrome c oxidase activity (by homology)</td>
</tr>
<tr>
<td>IPF630</td>
<td>CA6016</td>
<td>1:74</td>
<td>0.040</td>
<td>Nucleic acid binding</td>
</tr>
<tr>
<td>RPL28</td>
<td>CA1662</td>
<td>2:26</td>
<td>0.040</td>
<td>Structural constituent of ribosome</td>
</tr>
<tr>
<td>IPF19688</td>
<td>CA0301</td>
<td>2:10</td>
<td>0.039</td>
<td>Unknown function</td>
</tr>
<tr>
<td>IPF12942</td>
<td>CA3604</td>
<td>1:50</td>
<td>0.037</td>
<td>Δ-12 fatty acid desaturase (by homology)</td>
</tr>
<tr>
<td>IPF19968</td>
<td>CA3030</td>
<td>1:65</td>
<td>0.037</td>
<td>Putative cell wall protein of the PIR family</td>
</tr>
<tr>
<td>MXR1</td>
<td>CA0123</td>
<td>1:58</td>
<td>0.031</td>
<td>Methionine sulfoxide reductase</td>
</tr>
<tr>
<td>GAP6</td>
<td>CA4265</td>
<td>1:55</td>
<td>0.029</td>
<td>General amino acid permease (by homology)</td>
</tr>
<tr>
<td>IPF8336</td>
<td>CA2839</td>
<td>1:61</td>
<td>0.029</td>
<td>Unknown function</td>
</tr>
<tr>
<td>IPF407</td>
<td>CA5848</td>
<td>2:10</td>
<td>0.027</td>
<td>Unknown function</td>
</tr>
<tr>
<td>IPF19977</td>
<td>CA3211</td>
<td>1:68</td>
<td>0.024</td>
<td>Unknown function</td>
</tr>
<tr>
<td>RPL18</td>
<td>CA6079</td>
<td>1:55</td>
<td>0.024</td>
<td>Ribosomal protein L18B (large subunit) (by homology)</td>
</tr>
<tr>
<td>IFA14</td>
<td>CA5961</td>
<td>1:59</td>
<td>0.024</td>
<td>Unknown function</td>
</tr>
<tr>
<td>IPF10262</td>
<td>CA1160</td>
<td>1:65</td>
<td>0.023</td>
<td>Vacuole organization and biogenesis; phosphatidylethanolamine biosynthesis; actin cytoskeleton organization and biogenesis</td>
</tr>
<tr>
<td>IPF14981</td>
<td>CA2896</td>
<td>1:59</td>
<td>0.022</td>
<td>Glycerophosphodiester phosphodiesterase activity</td>
</tr>
<tr>
<td>IPF8904</td>
<td>CA3132</td>
<td>1:59</td>
<td>0.015</td>
<td>Unknown function</td>
</tr>
<tr>
<td>IPF3912</td>
<td>CA4960</td>
<td>2:68</td>
<td>0.001</td>
<td>Transcription factor activity</td>
</tr>
<tr>
<td>DDR48</td>
<td>CA4336</td>
<td>4:50</td>
<td>8.56 × 10⁻⁴</td>
<td>Stress protein (by homology)</td>
</tr>
</tbody>
</table>

*As compared with the wild-type control. †Student’s t-test.

Divided into functional groups, nine of these upregulated genes have no known or putative function (IPF5830, IPF3092, IPF11167, IPF19688, IPF8336, IPF407, IPF19977, IFA14 and IPF8904), five genes are known or putative
transporters (CTR1, GAP7, CDR1, HLA4 and GAP6), and five genes (RTA2, GUT2, MSS4, IPF14981 and IPF10262) are associated with the metabolism of inositol, phospholipids or glycerol, or have putative functions associated with vacuoles and membranes. Finally, four genes encode DNA-binding proteins or transcriptional regulators, with NRG1 being the gene most strongly upregulated of these. The gene most strongly upregulated overall was DDR48, encoding a putative hyphal-associated stress protein.

None of the orthologues of GLE1, IPK1 or IPK2 were significantly up- or downregulated, confirming the results obtained with RT-PCR in our heat-shock experiment.

**At least three genes in the genome of C. albicans encode phospholipases C**

Andaluz et al. (2001) described a gene encoding a phospholipase C (CaPI-PLC, renamed CaPLC2 in this study) (accession no. AJ277538) and a presumptive truncated homologous gene that differed in size by 145 bp, on chromosome 2 (named CaPLC3 in this study) in the genome of C. albicans, in addition to the CaPLC1 gene first cloned by Bennett et al. (1998). CaPLC2 was described as an ORF of 1029 bp encoding a protein of 343 amino acids (Andaluz et al., 2001) with no obvious signal peptide, transmembrane region or GPI-anchor-like sequences, suggesting that CaPLC2 encodes an intracellular phospholipase C. The deduced protein sequence of CaPLC2 is more similar to bacterial PI-specific phospholipases C, is very different from CaPlc1p (Andaluz et al., 2001) and has no homologous counterpart in S. cerevisiae. The higher similarity to bacterial phospholipases may be explained by the fact that CaPlc2p only consists of the X domain of mammalian phospholipases C. However, an alignment of the X domain of CaPlc1p and the entire CaPlc2p sequence showed only 8.5% similarity (Fig. 4).

CaPLC2 was identified on contig 6-2467 as ORF6.6631 in assembly 6 of the C. albicans genome at Stanford Genome Technology Center (http://www-sequance.Stanford.edu/group/candida) and CA4152/ORF19.5797 of CandidaDB (http://genolist.pasteur.fr/CandidaDB/) based on assembly 19. Its homologous gene CaPLC3 described by Andaluz et al. (2001) was identified as the Stanford ORF 6.5334 on contig 6-2402 in assembly 6 (CA3159/ORF19.1586 in CandidaDB) on chromosome 2. However, this gene was almost identical to CaPLC2 and showed no length differences. Cloning and sequencing of CaPLC2 and CaPLC3 in our hands confirmed that both genes were identical in size and differed only in two amino acids (amino acids 91 and 93: K and T in CaPlc2p, but N and S in CaPlc3p). We concluded that the reported differences in size (888 bp) were possibly due to a sequencing error (an additional adenosine in position 111 causing a frame shift). In contrast to the high similarity of the ORFs of CaPLC2 and CaPLC3, the 5’ and 3’ untranslated regions of the two genes differed dramatically. In summary, CaPLC2 and CaPLC3 represent two almost identical genes at different locations in the genome of C. albicans with no orthologue in S. cerevisiae.

**Fig. 4.** (a) DNA sequence of the 5’ untranslated region of CaPLC2 obtained in this study beginning with the ATG codon previously indicated as the start codon of CaPlc2p (AJ277538). Underlined are bases that differ from the published sequence causing frame shifts and a stop codon at position 140 bp. According to this sequence and the sequence in CandidaDB (http://www.pasteur.fr/recherche/unites/Galar-Fungal/) the putative ATG start codon (italics) of the ORF of CaPLC2 is located at position 145. (b) Protein sequence alignment of bacterial phospholipases C (BtPLC, Bacillus thuringiensis, accession no. P08954; LmPLC-A, Listeria monocytogenes, accession no. AAA69526), and CaPlc3p of C. albicans and the putative X domain (amino acids 386–785) of CaPlc1p. Amino acids that are conserved in all four sequences are bold/italics/underlined; amino acids conserved in three sequences are bold/italics. The two amino acids that differ between CaPlc2 and CaPlc3 are boxed.
In order to investigate whether the prokaryotic-type phospholipase genes CaPLC2 and CaPLC3 are distributed among other Candida species, we used CaPLC2 as a probe for a Southern blot analysis. Genomic DNA of different Candida species was digested with three restriction enzymes (EcoRI, BamHI and SalI). A CaPLC2 probe covering the whole ORF revealed no signal for Candida krusei and Candida glabrata, one band for Candida parapsilosis and Candida tropicalis and two bands for Candida albicans and Candida dublinensis, suggesting that only Candida species closely related to C. albicans contain genes similar to CaPLC2 or CaPLC3 (not shown).

The transcriptional profiles of CaPLC2 and CaPLC3 are similar

Several gene families have been identified in the genome of C. albicans (d’Enfert et al., 2005; Fradin & Hube, 2003). These families include gene members with high similarity and similar functions, but individual expression profiles. For example, expression of the SAP and PHR genes was shown to be tissue specific (Hube, 2004). Since CaPLC2 and CaPLC3, although highly similar in their ORFs, differed significantly in their promoter regions, we reasoned that these two genes might be differentially expressed in response to different environmental conditions.

The wild-type strain SC5314 was grown in different media (SD, YPD, YSer, SLAD or SG) and RNA samples from different time points were analysed by RT-PCR. To discriminate between CaPLC2 and CaPLC3 transcripts we used two gene-specific forward primers (24 bp in length, identical to regions upstream of the ATG start codon of CaPLC2 and CaPLC3) in combination with a single primer that recognized a sequence in the ORFs of both genes.

Under all conditions tested, both genes were expressed (Fig. 5). However, expression levels of CaPLC2 were higher under all conditions as compared with CaPLC3. Expression levels rose in the order SG > SLAD > YPD > YSer for CaPLC2, indicating a lower level of expression in hyphal cells (YSer) as compared with yeast cells (SG, SLAD, YPD) (it should be noted that under the conditions used we observed only yeast cells in SLAD medium, although SLAD medium is known to be a hyphal induction medium) (Fig. 5). Similarly, expression of CaPLC2 was higher in Lee’s medium at pH 4.5 (which supports yeast growth) as compared with Lee’s medium at pH 6.5 (which supports hyphal growth) (not shown). In contrast, the expression level of CaPLC3 was highest in SLAD as compared with all other growth conditions. Therefore, transcriptional regulation of CaPLC2 and CaPLC3 is at least partially different.

CaPLC2 and CaPLC3 are not essential for growth and virulence

In order to analyse the function of CaPLC2 and CaPLC3 we constructed mutants that lacked these genes. Mutants were generated using homologous recombination with only one disruption cassette in a multi-step procedure. Neither single mutants lacking CaPLC2 or CaPLC3 respectively nor double mutants lacking both genes had any growth defects in minimal or complex growth media, suggesting that CaPLC2 and CaPLC3 are not essential for growth.

Mutants lacking CaPLC2 or CaPLC3 or both genes showed no change in phenotype at reduced or elevated temperatures (25, 30, 37 or 43°C), in media with different carbohydrate sources (glucose, galactose or sucrose), under anaerobic conditions, in media with high NaCl or carbohydrate concentrations causing osmotic stress, or at different pH values (pH 5.7–8.0) as compared with the parental wild-type (CAF2-1). In addition, no growth differences were observed in SD medium supplemented with benomyl, calcofluor white, Congo red, nocodazole, hygromycin B, cycloheximide, EDTA, 5-flucytosine, tetracycline, amorpholine, SDS, butanol, 1-propanol or calcium (up to 300 mM) at elevated concentrations to achieve distinct effects of stresses.
to the cells. Furthermore, extracellular lipolytic activity was not reduced, as growth on blood or egg-yolk agar showed the same characteristic halo around colonies as compared with the wild-type, and serum-induced hyphal production was indistinguishable from the wild-type.

However, growth of the ΔCaplc2/ΔCaplc3 double mutant was inhibited at 30 °C in SD medium supplemented with 20 mM caffeine, or at 37 °C supplemented with 1 μg itraconazole ml⁻¹ (Fig. 6a) or in the presence of 880 μM hydrogen peroxide (not shown). Hyphal formation of the ΔCaplc2/ΔCaplc3 double mutant was delayed at 30 °C on CAA medium, and at 37 °C on M199 agar (Fig. 6a), on Spider medium and on YCB-BSA agar (not shown). Furthermore, the production of the characteristic white zone around colonies of the double mutant was delayed on YCB-BSA agar. None of the phenotypes observed for the double mutant were detected in the single mutants, suggesting that both genes have the potential to compensate for the loss of the homologous CaPLC gene and that the observed phenotypes are not due to a position effect of the URA3 marker.

When tested in a mouse model of systemic infection, the ΔCaplc2/ΔCaplc3 mutant did not show reduced survival rate or organ burdens (kidney and brain) as compared with the wild-type CAF2-1 (data not shown), suggesting that CaPLC2 and CaPLC3 are not essential for virulence.

**Delayed formation of hyphae is restored by extrachromosomally expressed CaPLC2**

In order to rescue the wild-type phenotype in mutants lacking CaPLC2 and CaPLC3, and to overexpress CaPLC2 or CaPLC3, both genes were cloned behind the PCK1 promoter of plasmid pBI-1 (Leuker et al., 1997). No obvious phenotypes were observed in media where the PCK1 promoter is induced (CAA medium, succinate medium or SGlycAc medium). However, when CaPLC3 was overexpressed at 30 °C on CAA medium in the ΔCaplc2/ΔCaplc3 double mutant, hyphal formation was similar to wild-type (CAF2-1) (Fig. 6b).

**DISCUSSION**

Secretd phospholipases have long been discussed as virulence factors of *C. albicans* (Ghannoum, 2000). Earlier studies suggested that this includes extracellular phospholipases C (Pugh & Cawson, 1977). The genes investigated in this study are the only genes encoding phospholipases C with conserved PLC motifs homologous to other known phospholipases C in the entire genome of *C. albicans* (http://www.Pasteur.fr/recherche/unites/Galar-Fungail/). None of the deduced sequences of *CaPLC1, CaPLC2* or *CaPLC3* contain N-terminal signal peptides, suggesting that these genes probably encode intracellular phospholipases which are not guided into the secretory pathway. Therefore, extracellular phospholipases C either do not exist in *C. albicans* or are encoded by enzymes with no obvious homologies to known phospholipases of this class. Intracellular phospholipases C are involved in a number of cellular processes. In this study we have produced mutants either lacking or overexpressing *CaPLC1*–3 and have analysed the consequence of these gene manipulations. While *CaPLC1* has an orthologue in the closely related yeast *S. cerevisiae*, *CaPLC2* and *CaPLC3* are unusual fungal phospholipases, more resembling bacterial phospholipases and having no counterpart in *S. cerevisiae*.

All attempts to generate a *CaPLC1* null mutant failed and disruption of the second allele was only possible in mutants
where the chromosomal CaPLC1 region had been duplicated, suggesting that CaPLC1 is an essential gene. This is further supported by the fact that the PI-Plc-specific inhibitor ET-18 completely blocks growth of C. albicans at higher concentrations. Thus, inhibition of CaPLC1 in C. albicans and perhaps other Candida species appears to be a possible therapeutic strategy.

Although PLC1 of S. cerevisiae has been shown to be essential in certain genetic backgrounds, other studies showed successful disruption of PLC1 in other strains. In an attempt to further show that CaPLC1 is in fact essential, we used the MET3 promoter of C. albicans, which has previously been used to study essential genes such as URA3 (Care et al., 1999). In our hands, addition of 2.5 mM methionine and cysteine repressed the MET3 promoter; however, transcript levels of CaPLC1 were still detectable and the Caplc1/pMET3-CaPLC1 mutant was still viable. Since the level of CaPLC1 transcripts in wild-type strains was also very low, we reasoned that the low level of transcription of CaPLC1 in the controllable mutant was sufficient for survival. Low transcript levels for PLC1 in wild-type cells had also been reported in S. cerevisiae (Flick & Thorner, 1993) and Bennett et al. (1998) were unable to detect CaPLC1 transcripts in C. albicans using Northern blot analysis. Therefore, the MET3 promoter seems to be too leaky to reduce transcription below an essential level for CaPLC1 and possibly other essential genes with low basal transcription levels in wild-type cells of C. albicans.

To our knowledge, until the present study, there has been no protocol described that would generally provide unambiguous evidence that a particular gene is essential for C. albicans. Similar to the approach of a conditional mutant using the MET3 promoter (Care et al., 1999), Roemer et al. (2003) set out to define C. albicans essential genes by deleting one allele and fusing a second allele to a tetracycline-repressible promoter, creating a ‘GRACE’ (gene replacement and conditional expression) strain. However, in addition to the creation of conditional mutants Davis et al. (2002) used another indirect approach to identify essential genes by using the UAU1 cassette, which permits selection of homozygous mutants from heterozygotes (Enloe et al., 2000).

Disruption of PLC1 in S. cerevisiae in a non-lethal genetic background revealed phenotypic responses such as defective utilization of carbon sources other than glucose, osmosensitivity and temperature sensitivity (Flick & Thorner, 1993; Payne & Fitzgerald-Hayes, 1993). The latter phenotype could be partially restored with exogenous Ca2+ ions, suggesting a role of PLC1 in Ca2+-signal transduction via IP3. The phenotypes of the conditional Caplc1/pMET3-CaPLC1 mutant of C. albicans were generally comparable to those observed for the PLC1 mutant from S. cerevisiae, suggesting that Caplc1 may have functions similar to PLC1 of S. cerevisiae. Growth of Caplc1/pMET3-CaPLC1 of C. albicans under repressed conditions included reduced growth (1) on media containing 5% (w/v) sorbitol or 1 M NaCl (causing increased osmotic stress), (2) on media with galactose as the sole source of carbon instead of glucose, (3) at elevated (43’) temperature or (4) at low (18°C) temperature. Furthermore, reduced growth on media with arginine as sole nitrogen source suggests that CaPLC1 may, like its orthologue in S. cerevisiae, be necessary for the production of IP messengers that modulate distinct nuclear processes via regulation of the ArgR–Mcm1 complex (Odom et al., 2000). A putative nuclear localization signal (NLS) (Odom et al., 2000; Payne & Fitzgerald-Hayes, 1993) rich in basic amino acids within the X and Y domain supports the view that CaPlc1p in C. albicans may in fact act in the nucleus. In addition to a putative role in nuclear mRNA export (Odom et al., 2000; York et al., 1999), sensitivity to nocodazole of the Caplc1/pMET3-CaPLC1 mutant may point to a role of CaPlc1p in chromosome segregation, since mutants lacking Pclp1 in S. cerevisiae showed chromosome mis-segregation when exposed to this drug (Lin et al., 2000). Therefore, CaPlc1p from C. albicans, like the Pclp1 counterpart in S. cerevisiae, seems to be involved in multiple cellular processes. This may explain why a genome-wide transcriptional profiling of the Caplc1/pMET3-CaPLC1 mutant did not produce a clear expression pattern reflecting distinct transcriptional responses to heat treatment. The observation that CaPLC1 seems to be an essential gene in C. albicans cannot simply be explained by the fact that CaPlc1p produces two essential second messengers, IP3 and DAG, as these molecules can also be produced by other enzymes (e.g. by Pld1p or Caplc2p or Caplc3p). Therefore, it must be concluded that CaPlc1p is the only enzyme in C. albicans which can produce IP3 and DAG under certain conditions at distinct cellular locations, or that CaPlc1p has further functions that cannot be compensated by other enzymes.

Incubation of C. albicans wild-type strains under conditions which repress the pMET3 promoter (addition of 2.5 mM methionine and cysteine) strongly inhibits the yeast-to-hyphal transition in media which are otherwise known to induce hyphal formation (such as media containing serum). Similarly, Shepherd et al. (1980) showed that higher concentrations of cysteine (>5 μM) inhibit germ tube formation. Therefore, the ΔCaplc1/pMET3-CaPLC1 conditional mutant was not suitable to investigate the effect of CaPLC1 on hyphal formation. In order to investigate the impact of reduced levels of CaPLC1 on hyphal formation, we analysed the growth of the heterozygous ΔCaplc1/CaPLC1 mutant in comparison with the CaPLC1/CaPLC1 wild-type strain under hyphal-inducing conditions. Similarly, Uhl et al. (2003) showed that heterozygous mutants produced in a large-scale loss-of-function genetic screen had defects in filamentous growth due to gene dosage effects.

Ansari et al. (1999) showed that Plc1p interacts with the receptor-like membrane protein Gpr1p as a component of the nitrogen-signalling pathway for formation of pseudohyphae in S. cerevisiae. Furthermore, Gpr1p is associated with the regulator protein Gpa2 at the initial steps of signal
transduction (Ansari et al., 1999). Based on these results it was suggested that Plc1p acts in two filamentation pathways in S. cerevisiae, the AMP- and the Ras2-controlled (MAPK) pathway. The cAMP and the MAPK pathways in C. albicans are known to be associated with the yeast-to-hyphal transition. In fact, mutants of C. albicans lacking either one copy of CaPLC1, or both CaPLC2 and CaPLC3, had reduced abilities to produce hyphae on certain media (e.g. Spider medium). The fact that the ∆CaPlc1/CaPLC1 heterozygote had reduced ability to grow on serum agar plates may be related to modified Ca^{2+}-mediated signal transduction and increased sensitivity to serum (Sanglard et al., 2003). However, addition of external Ca^{2+} ions at several concentrations did not alter the phenotype of the heterozygous mutant (not shown). Sanchez-Martinez & Perez-Martin (2002) showed that mutants lacking GPA2 of C. albicans also failed to produce hyphae on Spider medium and concluded that Gpa2p acts in the MAPK pathway. However, a direct interaction between Gpa2 and Gpr1 or any of the proteins CaPlc1–3p with proteins of the MAPK pathway in C. albicans has yet not been shown.

The exact role of CaPLC2 and CaPLC3 of C. albicans remains unclear. The function of both genes must be clearly different from CaPLC1, as neither CaPLC2 nor CaPLC3 can compensate for the loss of CaPLC1. The fact that these two genes are unique for C. albicans and do not exist in S. cerevisiae may suggest a role in adaptation to the human host. However, both genes are dispensable for virulence in one model of infection (this study) and for interaction with macrophages (Knechtle et al., 2005). It also remains a mystery why C. albicans provides two almost identical copies of this unusual type of PLC gene in eukaryotes. One possible explanation would be a differential regulation of CaPLC2 and CaPLC3 under different conditions, as has been shown for other gene families in C. albicans (Fradin & Hube, 2003). However, although the promoter regions of both genes are very different, the expression pattern was similar under all conditions tested and both genes seem to be constitutively expressed in vitro. It is possible that CaPLC2 and CaPLC3 are differentially expressed under certain conditions in vivo and that the expression under these conditions is crucial for survival of C. albicans.

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