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 *Sacharomyces 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 $\Delta Caplc1/1$ *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.

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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)

Abbreviations: DAG, 1,2-diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PI, phosphoinositide; PI-Plc, PI-specific phospholipase C.

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.

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 mis-segregation, 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 Ca^{2+} ions, which supported the view that *PLC1* is involved in Ca^{2+} -signal transduction of *S. cerevisiae*. *PLC1* was shown to be essential for glucose-stimulated PI turnover and subsequent activation of plasma-membrane H^+ -ATPases (Cocchetti *et al.*, 1998), and mutants lacking *PLC1* failed to regulate intracellular Ca^{2+} 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 CAI4 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) $(\text{NH}_4)_2\text{SO}_4$, 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 [1 l bouillon [0.3 % (w/v) NaCl, 0.3 % (w/v) $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 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-glycero-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 $\Delta\text{Caplc1}/\text{pMET3-}\text{CaPLC1}$, 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 $\text{Caplc1}/\text{CaPLC1}$ 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, CAF2-1 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 OD_{600} of 0.6. The culture was then divided into 40 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 until the appropriate OD_{600} was reached (see Fig. 5). Aliquots (10 ml) were harvested for RNA extraction. For SLAD [6.7 g l^{-1} YNB (Difco) without amino acids and ammonium sulfate plus 0.05 mM $(\text{NH}_4)_2\text{SO}_4$] and YPD plus 5 % (v/v) fetal calf serum (YSer), 2×10^7 cells ml^{-1} were inoculated from a preculture grown in SD at 37 °C and 25 ml aliquots were harvested for RNA extraction. For induction of the PCK promoter we used SGlycAc [SD without glucose but with 2 % (w/v) potassium acetate and 3 % (v/v) glycerol] and succinate medium (B-medium) (Leuker *et al.*, 1997; Stoldt *et al.*, 1997).

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 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 *PvuII* 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 $\Delta\text{Caplc1}/\text{CaPLC1}$ 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 URA-1 and URA-2 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 $\Delta\text{Caplc1}/\text{CaPLC1}$ mutant.

Construction of pDS952. A 4299 bp PCR fragment containing bp 9821 to bp 14120 of contig 6-2335 flanked by *XbaI* and *XhoI* sites was cloned into pBluescript. A second PCR fragment was produced

Table 1. Primers

Primer	Sequence (5'–3')
PLC1-1	ATG TTG GAA TCA TTA AAT CGT C
PLC1-2	GTA CAA TTG ATC TTG ATT CAT C
PLC1-3	TTT GGG ATT CAT TAG CTC AAG G
PLC1-4	TTG TAT CGA CAT TGC TAC TGC
URA-1	GAT CAA GCT TGG ATG GTA TAA ACG GAA
URA-2	GAT CAA GCT TCT AGA AGG ACC ACC TTT G
PLC2-0	CAA TCG ATT GGT TTC TAT TTC AAT
PLC2-1	CAA AAC TTG GTT AAA GGA TAT TGA
PLC2-3	GTT CAT CTT GAA CAC CAA ATC
PLC2-4	ACA CAA TGA CAG TCT CCG AAG AA
PLC2-5	ATG AAG CTC TAT TAC TCG CAT CTT
PLC2-6	TTA GTA TCA ATT AAA GCT TCA GGC
PLC2-7	GAT CGG TAC CTG ATG CTA GAG GTA
PLC2-8	GAT CAA GCT TGA CAG TCT CCG AAG
PLC3-1	CAC TGA CAT ATA ATA CAT TAA TTC
PLC2-9	AGA TCT ATG AAG CTC TAT TAC TCG
PLC2-10	AGA TCT TGC TTA ATG CGA CAA ATA
PLC3-2	AGA TCT ATG GTC GAC TAC AAA ACT
PLC3-3	AGA TCT AGT TGT CAA CTT TGA AGC
EFB1-1	AGT CAT TGA ACG AAT TCT TGG CTG
EFB1-2	TTC TTC AAC AGC AGC TTG TAA GTC
ACT1-1	ACC GAA GCT CCA ATG AAT CCA
ACT1-2	GGA TGG ACC AGA TTC GTC GTA
GLE1-1	GAG GAA CGC AGA CGC CAA GAA
GLE1-2	GAT CAT TCC AAT CAC TTC AGT ACT
IPK1-1	AAG ATC ACT TCA CCA GAA GAT TG
IPK1-2	GTT TAG AGA ACA AGT TCG ACA GTA
IPK2-3	CTC AAG TTC AAC TGC ACG ATC AA
IPK2-4	CTT ATG GTC ATC AGC ATC ATT TAT
T7	TAA TAC GAC TCA CTA TAG GG
PLC1-5	TAT TAT CTA GTT ATT CAA CTC CAT ATC GTG AAA TCA CCC AAA CCC AAA CTA ACT ATT ATT GGA TGG TAT AAA CGG AAA CA
PLC1-6	GTC TTA TAA TGA ATG ATG ATG TAG AAC TAC TAT TAG ATT TAT TAT TTT CAT TAT CTT TCT AGA AGG ACC ACC TTT GAT TG
PLC1-7	CGG GAT TCC GAT GTT GGA ATC ATT AAA
PLC1-8	CCA ATG CAT TGG CTG CAG GCT TTT CTG TTG TTG CTG
pMET1	TTC AAC TTC CAA GTG TTG TCA C
PLC1-9	TTG TAT CGA CAT TGC TAC TGC

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.

Construction of cassette 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

URA3 marker as described by Wilson *et al.* (1999). A primer pair was designed in which each primer overlapped both the *CaPLC1* and *URA3* genes. Primer PLC1-5 shared 58 bp and 22 bp homology to the *CaPLC1* and *URA3* genes, respectively and PLC1-6 had 57 bp and 23 bp homology to the *CaPLC1* and *URA3* genes, respectively (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 *Bam*HI and a *Pst*I 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 *Bam*HI/*Pst*I sites of pCaDis (Care *et al.*, 1999) to give pIM14. pIM14 was linearized with *Cl*aI and transformed into the *Ura*⁻ heterozygous Δ *Caplc1*/*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 *Hind*III site, while integration into the disrupted allele resulted in an amplicon of 3 kb without a *Hind*III 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, PLC3-2 and PLC3-3. PCR products were ligated into pGEM-T or 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 *Hind*III restriction site downstream of the insert was deleted by linearizing the plasmid with *Hind*III, 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 *PLC2* 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 pBI-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 *Bgl*II site of pBI-1, generating pDK-16 and pDK-18. Integration of *CaPLC2* and *CaPLC3* downstream of the *PCK1* promoter was confirmed by analysing 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 (Felk *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 (Felk *et al.*, 2002). For RT-PCR, 1.5 µg RNA was DNase treated and cDNA synthesized (Felk *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_Fungail/arrays.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 (Δ *plc2*/ Δ *plc3*, 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 CaPlc1p (amino acids 718–790) points to a nuclear localization (Dingwall & Laskey, 1991).

In order to investigate the function of CaPlc1p 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 *Hind*III 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 (Yesland & 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 cassette), 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, Δ *Caplc1/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 Δ *Caplc1/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 phenotypic screening.

Phenotypic screening of Δ *Caplc1/pMET3-CaPLC1*

A first phenotypic screening of the Δ *Caplc1/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 in media with carbon sources other than glucose. Under these conditions, Δ *Caplc1/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.

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 *S. cerevisiae* is necessary for the transcriptional control of the ArgR–Mcm1 complex (Odom *et al.*, 2000), we also tested growth of the $\Delta Caplc1/pMET3-CaPLC1$ 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 $\Delta Caplc1/pMET3-CaPLC1$ conditional mutant under conditions that repress the *MET3* promoter. The wild-type strain CAF2-1 and the $\Delta Caplc1/pMET3-CaPLC1$ mutant were grown for 1 day in liquid SD medium supplemented with 2.5 mM methionine and cysteine to repress the *MET3* promoter. Cells were counted, diluted and 5 μ l 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 $\Delta Caplc1/CaPLC1$ mutant

Since we observed growth phenotypes for the $\Delta Caplc1/pMET3-CaPLC1$ mutant under conditions which repressed the *MET3* promoter but which did not completely block transcription of the *CaPLC1* gene, we reasoned that the heterozygous $\Delta Caplc1/CaPLC1$ 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 $\Delta Caplc1/CaPLC1$ 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 μ g ml⁻¹) or hydrogen peroxide (880 μ M), 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 $\Delta Caplc1/CaPLC1$ colonies (Fig. 3).

The PI-Plc-specific inhibitor ET-18 blocks growth of *C. albicans*

Since gene disruption experiments suggested that *CaPLC1* is an essential gene, we analysed the effect of the PI-Plc-specific inhibitor 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine (ET-18) on growth of *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 μ M 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 μ M, ET-18 caused 80 % inhibition of hyphal formation (Table 2) and at ET-18 concentrations higher than 200 μ M (not shown) growth was completely prevented, suggesting that PI-Plcs in *C. albicans* are essential for growth.

Construction of a conditional *CaPLC1* mutant in a *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 *PLD1* (Hube *et al.*, 2001) we questioned whether a

Fig. 3. Phenotypes under hyphal-inducing conditions of the heterozygous mutant ($\Delta Caplc1/CaPLC1$; upper panel of each pair) as compared with the wild-type CAF2-1 (lower panel of each pair). Serial cell dilutions from an overnight SD liquid culture were dropped on each medium. Cells were incubated on SD and YPD (30 °C and 37 °C for 2 days), on YCB-BSA (30 °C for 8 days and 37 °C for 4 days), on Spider medium and on blood agar (30 °C for 5 days and 37 °C for 8 days).

disruption of *PLD1* in addition to downregulation of *CaPLC1* transcripts might have a synergistic effect. Therefore, we

Table 2. The PI-PLC-specific inhibitor ET-18 blocks growth of *C. albicans*

Means $\pm$ SD from three independent samples at each time point and ET-18 concentration are shown.

Time (h)	Percentage of hyphal-forming cells			
	0 μ M ET-18	10 μ M ET-18	20 μ M ET-18	200 μ M ET-18
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
1	41.4 $\pm$ 4.3	31.1 $\pm$ 1.9	21.9 $\pm$ 6.0	8.1 $\pm$ 1.8
2	68.3 $\pm$ 11.0	43.7 $\pm$ 4.4	30.7 $\pm$ 1.8	20.9 $\pm$ 1.9
3	79.3 $\pm$ 3.6	45.4 $\pm$ 4.2	30.3 $\pm$ 3.2	18.2 $\pm$ 3.6
4	85.4 $\pm$ 2.5	48.9 $\pm$ 0.4	32.3 $\pm$ 2.1	16.3 $\pm$ 2.5
5	96.7 $\pm$ 2.9	53.9 $\pm$ 2.5	38.4 $\pm$ 8.8	13.2 $\pm$ 0.6

disrupted one allele of *CaPLC1* in the *pld1/pld1* mutant and replaced the native *CaPLC1* promoter with the *MET3* promoter as described above. In addition to the above-described phenotypes for the $\Delta Caplc1/pMET3-CaPLC1$ mutant, the conditional *pld1/pld1* $\Delta Caplc1/pMET3-CaPLC1$ mutant showed enhanced osmosensitivity at 1 M NaCl (not shown).

Transcriptional analysis of *CaPLC1* and genes putatively linked to *CaPLC1* function

Phenotypic screening of mutants lacking *PLC1* in *S. cerevisiae* (Odom *et al.*, 2000) and *C. albicans* (this study) revealed sensitivity to different environmental stresses. Therefore, we questioned whether *CaPLC1* is regulated in response to such stresses. In *S. cerevisiae* the products of the *PLC1*, *GLE1*, *IPK1* and *IPK2* (*ARG82*) genes are known to be involved in nuclear mRNA export (York *et al.*, 2001) and may be co-regulated. A mutant lacking *GLE1* in *S. cerevisiae* showed temperature sensitivity similar to mutants lacking *PLC1*. Therefore, we examined the transcript level of *CaPLC1* and the orthologous genes of *GLE1*, *IPK1*, *IPK2* in *C. albicans* wild-type cells (*CaGLE1*, IPF15261, CA2378; *CaIPK1*, IPF10566, CA0280; *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 *CaPLC1*, *GLE1* and *IPK2* seemed moderately increased at 42 °C and for *CaPLC1* moderately reduced in medium containing cadmium (Fig. 1).

Genome-wide transcriptional profiling of the conditional $\Delta Caplc1/pMET3-CaPLC1$ mutant

PI turnover due to the hydrolytic activity of phospholipase C produces not only DAG, but also IP₃, another intracellular second messenger that stimulates release of Ca²⁺, activates calmodulin-dependent enzymes and is a substrate for inositol polyphosphates. Therefore, reduced levels of *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 1×10^7 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 (*GDH3*, *MET18*, *IPF12399* and *CDC46*) were found to be down-regulated and 41 genes upregulated (Table 3).

Table 3. Genes upregulated in the conditional $\Delta Caplc1/pMET3-CaPLC1$ 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

Gene name	Systematic name	Normalized fold upregulated*	P-value†	Known or putative function
<i>SFT1</i>	CA5433	1.89	0.099	SNARE-like protein (by homology)
<i>CTR1</i>	CA1496	1.62	0.099	Copper transport protein
<i>IPF15013</i>	CA2761	1.51	0.097	Pyruvate decarboxylase regulatory protein (by homology)
<i>IPF5830</i>	CA4855	1.54	0.095	Unknown function
<i>NBN1</i>	CA4688	1.72	0.094	Involved in chromatin remodelling and possibly transcription regulation (by homology)
<i>NRG1</i>	CA5289	1.71	0.093	Transcriptional repressor
<i>GUT2</i>	CA3566	1.78	0.086	Glycerol-3-phosphate dehydrogenase, mitochondrial (by homology)
<i>IPF10564</i>	CA3328	1.50	0.086	Oxidoreductase, disulfide-oxidoreductase
<i>IPF17676</i>	CA0411	1.70	0.083	Similar to <i>S. cerevisiae</i> Cst13p involved in chromosome stability (by homology)
<i>RTA2</i>	CA3607	2.45	0.077	Phospholipid-translocating ATPase activity
<i>IPF3092</i>	CA4658	1.54	0.077	Unknown function
<i>CRH11</i>	CA0375	2.17	0.075	Probable membrane protein (by homology); endopeptidase, hydrolase
<i>GAP7</i>	CA0757	1.52	0.072	General amino acid permease
<i>CDR1</i>	CA6066	1.77	0.072	ABC transporter
<i>MSS4</i>	CA0623	1.90	0.068	Phosphatidylinositol-4-phosphate 5-kinase (by homology)
<i>IFM3</i>	CA3742	1.98	0.065	2-Hydroxyacid dehydrogenase (by homology), glycerate-formate-dehydrogenase-like
<i>SCW1</i>	CA0156	1.53	0.061	Glucanase (by homology)
<i>RBT2</i>	CA3957	1.91	0.059	Repressed by TUP1 protein 2; Rbt2p, ferric reductase (by homology)
<i>HOL4</i>	CA4204	1.67	0.058	Member of major facilitator superfamily multidrug-resistance protein subfamily 1 (by homology)
<i>ECM331</i>	CA2181	2.22	0.055	Involved in cell wall biogenesis and architecture (by homology)
<i>IPF11167</i>	CA0759	1.56	0.053	Unknown function
<i>FRE7</i>	CA5621	2.42	0.047	Ferric reductase transmembrane component (by homology)
<i>IPF4999</i>	CA2874	2.23	0.045	DNA-binding, two-component response-regulator
<i>COX19</i>	CA0768	1.53	0.043	Protein required for cytochrome <i>c</i> oxidase activity (by homology)
<i>IPF630</i>	CA6016	1.74	0.040	Nucleic acid binding
<i>RPL28</i>	CA1662	2.26	0.040	Structural constituent of ribosome
<i>IPF19688</i>	CA0301	2.10	0.039	Unknown function
<i>IPF12942</i>	CA3604	1.50	0.037	Δ -12 fatty acid desaturase (by homology)
<i>IPF19968</i>	CA3030	1.65	0.037	Putative cell wall protein of the PIR family
<i>MXR1</i>	CA0123	1.58	0.031	Methionine sulfoxide reductase
<i>GAP6</i>	CA4265	1.55	0.029	General amino acid permease (by homology)
<i>IPF8336</i>	CA2839	1.61	0.029	Unknown function
<i>IPF407</i>	CA5848	2.10	0.027	Unknown function
<i>IPF19977</i>	CA3211	1.68	0.024	Unknown function
<i>RPL18</i>	CA6079	1.55	0.024	Ribosomal protein L18B (large subunit) (by homology)
<i>IFA14</i>	CA5961	1.59	0.024	Unknown function
<i>IPF10262</i>	CA1160	1.65	0.023	Vacuole organization and biogenesis; phosphatidylethanolamine biosynthesis; actin cytoskeleton organization and biogenesis
<i>IPF14981</i>	CA2896	1.59	0.022	Glycerophosphodiester phosphodiesterase activity
<i>IPF8904</i>	CA3132	1.59	0.015	Unknown function
<i>IPF3912</i>	CA4960	2.68	0.001	Transcription factor activity
<i>DDR48</i>	CA4336	4.50	8.56×10^{-4}	Stress protein (by homology)

*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*, *HOL4* 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-sequence.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-Fungail/>) 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 *Sall*). 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-fluorcytosine, tetracycline, amorolfine, SDS, butanol, 1-propranolol or calcium (up to 300 mM) at elevated concentrations to achieve distinct effects of stresses

Fig. 5. Transcript profile of *CaPLC2* and *CaPLC3* under different growth conditions. *C. albicans* strain SC5314 was grown in SLAD, YSer, YPD or SG medium and RNA samples from different time points or at different optical densities were used for RT-PCR analysis. Transcripts were amplified with 30 (left panels) or 35 (right panels) cycles using primer pairs that detect either *CaPLC2* or *CaPLC3* or transcripts from both genes. Primers detecting transcripts of *ACT1* and *EFB1* were used as internal standards (20 and 25 cycles) (see Fig. 1). Samples were taken from SLAD medium at 2 h and 4.25 h, from YSer medium at 0.5 h, 1 h and 3.5 h, from YPD medium at OD₆₀₀ 0.8 (1) and OD₆₀₀ 1.7 (2), from SD medium at OD₆₀₀ 0.7 (1), and from SG medium at OD₆₀₀ 1.0 (1) and OD₆₀₀ 1.6 (2).

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 $\Delta Caplc2/\Delta Caplc3$ double mutant was inhibited at 30 °C in SD medium supplemented with 20 mM caffeine, or at 37 °C supplemented with 1 μ g itraconazole ml^{-1} (Fig. 6a) or in the presence of 880 μ M hydrogen peroxide (not shown). Hyphal formation of the $\Delta Caplc2/\Delta Caplc3$ 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 $\Delta Caplc2/\Delta Caplc3$ 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 $\Delta Caplc2/\Delta Caplc3$ double mutant, hyphal formation was similar to wild-type (CAF2-1) (Fig. 6b).

DISCUSSION

Secreted 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

Fig. 6. Reduced growth of the $\Delta Caplc2/\Delta Caplc3$ double mutant in SD medium containing 20 mM caffeine or 1 μ g itraconazole ml^{-1} (a), and delayed hyphal formation in M199 (a) and CAA medium (b). The delayed hyphal formation of the $\Delta Caplc2/\Delta Caplc3$ mutant can be abolished with extrachromosomal *CaPLC3* driven by the *PCK*-promoter on pBI-1 (b).

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 Ca^{2+} ions, suggesting a role of *PLC1* in Ca^{2+} -signal transduction via IP_3 . 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°C) 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 Plc1p in *S. cerevisiae* showed chromosome mis-segregation when exposed to this drug (Lin *et al.*, 2000). Therefore, CaPlc1p from *C. albicans*, like the Plc1p 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, IP_3 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 IP_3 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 $\Delta\text{Caplc1/pMET3-CaPLC1}$ 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 $\Delta\text{Caplc1/CaPLC1}$ 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 pseudo-hyphae 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 cAMP- 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 Δ *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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