Genetic characterization of a phospholipase C gene from *Candida albicans*: presence of homologous sequences in *Candida* species other than *Candida albicans*

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Phospholipase C (PLC) enzymes are essential in regulating several important cellular functions in eukaryotes, including yeasts. In this study, PCR was used to identify a gene encoding PLC activity in *Candida albicans*, using oligonucleotide primers complementary to sequences encoding highly conserved amino acid regions within the X domains of previously characterized eukaryotic phospholipase C genes. The nucleotide sequence of the *C. albicans* gene, *CAPLC1* (2997 bp), was determined from a recombinant clone containing *C. albicans* 132A genomic DNA; it encoded a polypeptide of 1099 amino acids with a predicted molecular mass of 124.6 kDa. The deduced amino acid sequence of this polypeptide (*CAPLC1*) exhibited many of the features common to previously characterized PLCs, including specific X and Y catalytic domains. The *CAPLC1* protein also exhibited several unique features, including a novel stretch of 18–19 amino acid residues within the X domain and an unusually long N-terminus which did not contain a recognizable EF-hand Ca^{2+} -binding domain. An overall amino acid homology of more than 27% with PLCs previously characterized from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* suggested that the *CAPLC1* protein is a δ -form of phosphoinositide-specific PLC (PI-PLC). PLC activity was detected in cell-free extracts of both yeast and hyphal forms of *C. albicans* 132A following 7 h and 24 h growth using the PLC-specific substrate *p*-nitrophenylphosphorylcholine (*p*-NPPC). In addition, *CAPLC1* mRNA was detected by reverse transcriptase PCR in both yeast and hyphal forms of *C. albicans* 132A at the same time intervals. Expression of *CAPLC1* activity was also detected in extracts of *Escherichia coli* DH5 α harbouring plasmids which contained portions of the *CAPLC1* gene lacking sequences encoding part of the N-terminus. Southern hybridization and PCR analyses revealed that all *C. albicans* and *Candida dubliniensis* isolates examined possessed sequences homologous to *CAPLC1*. Sequences related to *CAPLC1* were detected in some but not all isolates of *Candida tropicalis*, *Candida glabrata* and *Candida parapsilosis* tested, but not in the isolates of *Candida krusei*, *Candida kefyr*, *Candida guilliermondii* and *Candida lusitanae* examined. This paper reports the first description of the cloning and sequencing of a PLC gene from a pathogenic yeast species.

Keywords: *Candida albicans*, phosphoinositide-specific phospholipase C, *CAPLC1*, X domain

Abbreviations: PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; *p*-NPPC, *p*-nitrophenylphosphorylcholine.

The EMBL accession numbers for the nucleotide sequences reported in this paper are Y13975 (*Candida albicans* 132A) and Y14062 (*Candida dubliniensis* CD48-I).

INTRODUCTION

The incidence of opportunistic infections caused by species of the genus *Candida* has increased dramatically over the last two decades, especially in immunocompromised patients. These include individuals infected with the human immunodeficiency virus (HIV), patients with AIDS and those undergoing chemotherapy or immunosuppressive therapy. In HIV-infected patients candidal infections are largely confined to the oral cavity and the oesophagus, whereas systemic candidosis is more common in patients with neutropaenia (Coleman *et al.*, 1997). *Candida albicans* is the predominant *Candida* species responsible for candidosis, although the incidence of infection caused by other species of *Candida* has increased (Sullivan *et al.*, 1996).

Phospholipases play important roles in regulating a variety of cellular functions in mammalian cells and in lower eukaryotes, including yeasts and slime moulds. Five kinds of phospholipase activity have been described for *C. albicans*, including phospholipase A (Costa *et al.*, 1968; Pugh & Cawson, 1975, 1977; Price & Cawson, 1977; Barrett-Bee *et al.*, 1985); phospholipase B (Banno *et al.*, 1985; Ibrahim *et al.*, 1995, 1996); phospholipase C (PLC) (Costa *et al.*, 1968; Pugh & Cawson, 1977); lysophospholipase (Pugh & Cawson, 1975, 1977; Price & Cawson, 1977; Banno *et al.*, 1985) and lysophospholipase-transacylase (Takahashi *et al.*, 1991; Mirbod *et al.*, 1995; Ibrahim *et al.*, 1995). Phosphoinositide-specific phospholipase C enzymes (PI-PLCs) play a pivotal role in receptor-linked signalling at the plasma membrane of eukaryotic cells. Hydrolysis of inositol phospholipids, particularly phosphatidyl-inositol-4,5-bisphosphate (PIP₂) by PI-PLCs results in the formation of secondary messenger molecules, *sn*-1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃), which are important in the regulation of many cellular functions (Berridge, 1993). IP₃ causes the releases of Ca²⁺ ions from internal reserves and DAG activates protein kinase C (PKC). Calcium mobilization and PKC activation are essential for many cellular activities, including secretion, cell growth and proliferation (Nishizuka, 1992; Mitchell, 1992). PI-PLCs have been categorized into three subfamilies, β -, γ and δ , with two to four isoforms in each. These enzyme subfamilies each contain two well-conserved amino acid regions, designated the X and Y domains, which are essential for enzyme activity (Ellis *et al.*, 1993; Yagisawa *et al.*, 1994). Although the overall similarity between subfamilies is low, enzymes from each of the three subfamilies can be distinguished by their primary amino acid structure and molecular size (Rhee *et al.*, 1989). The δ isoenzymes are the smallest and simplest PI-PLCs as they contain only sequences common to all PI-PLC subfamilies and are common to all eukaryotes, from yeasts and moulds to plants and mammals. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* δ -type PI-PLCs are essential for growth and they are thought to be involved in relaying different nutritional and stress-related signals (Yoko-o *et al.*, 1993; Fankhauser *et al.*, 1995).

To date there have been no published reports describing the genetic characterization of determinants encoding PI-PLC enzymes from any *Candida* species. Because PI-PLCs have a central function in plasma membrane receptor-linked signalling in eukaryotic cells, including yeasts and slime moulds, it is highly likely that these enzymes perform a similar function in *C. albicans* and other *Candida* species. The purpose of the present study was to clone and characterize a PI-PLC genetic determinant from *C. albicans* and to investigate whether similar determinants are present in a number of other *Candida* species. Here we report the molecular cloning and characterization of a PI-PLC genetic determinant (CAPLC1) from *C. albicans* and demonstrate expression of the gene in both the yeast and hyphal forms of the organism. The deduced amino acid sequence encoded by CAPLC1 exhibits organizational features common to a wide variety of PI-PLC proteins from a diverse range of eukaryotic species but also shows unique features. We also report the presence of sequences homologous to CAPLC in several *Candida* species other than *C. albicans*, including *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*.

METHODS

Candida strains, clinical isolates and culture media. The reference *Candida* strains used in this study were *C. albicans* 132A (Gallagher *et al.*, 1992); *C. dubliniensis* CD48-I and CD46 (Moran *et al.*, 1997); *C. lusitanae* NCPF 3156; and *C. krusei* NCPF 3321; the last two strains were obtained from the National Collection of Pathogenic Fungi (UK). The oral isolates used in the study included 15 *C. albicans*, 10 *C. dubliniensis* (Moran *et al.*, 1997), 11 *C. glabrata*, five *C. tropicalis*, two *C. parapsilosis* and one isolate each of *C. kefyr* and *C. guilliermondii*, each from separate AIDS patients with oral candidosis. Twelve *C. albicans* oral carriage isolates from HIV-negative normal healthy individuals were also included. The yeasts were routinely grown on Potato Dextrose agar (PDA, Oxoid) at pH 5.6 for 48 h at 37 °C. For liquid culture, *Candida* isolates and strains were routinely grown in Yeast Peptone Dextrose broth [YPD; (l⁻¹) 10 g yeast extract (Oxoid), 20 g peptone (Difco), 20 g glucose, pH 5.5] at 37 °C in an orbital incubator (Gallenkamp) set at 180 r.p.m. The identity of each isolate was confirmed by their substrate assimilation patterns using the API ID 32C yeast identification system (bioMérieux), according to the manufacturer's instructions. The assimilation profiles of each isolate were examined on at least three separate occasions using different batches of ID 32C kits. Germ tube and chlamydospore tests were carried out on each isolate using the methods described by Sullivan *et al.* (1995). The identity of *C. dubliniensis* isolates was confirmed as described previously (Sullivan *et al.*, 1995, 1996; Moran *et al.*, 1997).

Bacterial strains and culture media. *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used as the host strain for plasmid pBluescript II KS(–) (Stratagene) and its recombinant derivatives and was maintained on LB agar (Sambrook *et al.*, 1989) containing 100 μ g ampicillin ml⁻¹ (LA medium). Plasmid DNA was transformed into competent CaCl₂-treated DH5 α cells using the procedure described by Sambrook *et al.* (1989). Following transformation, cells were plated immediately on to LA medium containing 100 μ g X-Gal (Boehringer Mannheim) ml⁻¹ and 1 mM IPTG (Boehringer Mannheim) and trans-

formants were selected using blue-white selection as described by Sambrook *et al.* (1989). For liquid culture, strains harbouring plasmids were routinely grown in LB broth (Sambrook *et al.*, 1989) containing 100 µg ampicillin ml⁻¹ at 37 °C in an orbital incubator (Gallenkamp) set at 180 r.p.m. Derivatives of DH5α harbouring recombinant plasmids pDB008, pDB009, pDB010, pDB016 or pDB017 were grown in LB broth both with and without X-Gal (100 µg ml⁻¹) and IPTG (1 mM) for use in PLC expression studies and were routinely maintained on LA agar medium. Large- and small-scale *E. coli* plasmid DNA preparations were performed as described by Sambrook *et al.* (1989).

The procedures used for the preparation of lambda phage stocks and the isolation of lambda phage DNA from *E. coli* phage lysates were as described by Sambrook *et al.* (1989). *E. coli* strain LE 392 (Sambrook *et al.*, 1989) and its P2 phage lysogenic derivative, P2 393 (Sambrook *et al.*, 1989), were used for propagating the bacteriophage lambda cloning vector EMBL3 (Promega) and its recombinant derivatives, respectively, on LB medium supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose as described by Sambrook *et al.* (1989). For liquid culture, organisms for phage infection were routinely grown in LB medium containing 10 mM MgSO₄ and 0.2% (w/v) maltose at 37 °C in an orbital incubator (Gallenkamp) set at 180 r.p.m.

Chemicals, enzymes and radioisotopes. Analytical-grade or molecular-biology-grade chemicals were purchased from Sigma, BDH or Boehringer Mannheim. [α -³²P]dATP (3000 Ci mmol⁻¹; 110 TBq mmol⁻¹) was purchased from Amersham. Restriction endonucleases, T4 DNA ligase (3000 Weiss units ml⁻¹), Klenow DNA polymerase (5000 units ml⁻¹), RNase-free DNase (10000 units ml⁻¹) and *Taq* DNA polymerase (5000 units ml⁻¹) were purchased from Promega or from Boehringer Mannheim and were used according to the manufacturer's instructions.

DNA hybridization. Probe DNA used in Southern hybridization experiments was labelled with [α -³²P]dATP (3000 Ci mmol⁻¹; 110 TBq mmol⁻¹) by the use of a random hexanucleotide primer labelling kit (Prime-a-Gene, Promega), which was used according to the manufacturer's instructions. Membrane filters containing bound DNA were processed under conditions of high stringency (>60 °C) as described by Sambrook *et al.* (1989), using a rotary hybridization oven (Hybaid).

Isolation of genomic DNA for hybridization analysis and PCR. Total cellular DNA from *Candida* isolates was prepared according to the method described by Sullivan *et al.* (1995). Restriction-endonuclease-generated fragments were separated by electrophoresis through 0.8% (w/v) horizontal agarose gels using 0.5 × TBE buffer (Sambrook *et al.*, 1989) as the electrophoresis buffer for 15 h at 2 V cm⁻¹. Following electrophoresis, DNA fragments were transferred to MagnaGraph nylon membrane filters (MSI) by the method of Southern (1975). Hybridization experiments were repeated on at least two occasions with separate preparations of genomic DNA.

PCR. Two degenerate PCR primers, termed CAPHO5 and CAPHO6, respectively (Table 1), were designed which were complementary to DNA sequences encoding two highly conserved amino acid regions within the X domain of 22 previously characterized PLC proteins from seven separate eukaryotic species (Table 2). One of these degenerate primers (CAPHO5) consisted of a 26 bp forward primer complementary to a sequence encoding the conserved amino acid sequence SSHNTYL, which also included the 6 bp recognition sequence for the restriction endonuclease *Eco*RI at the 5' end. The

reverse primer (CAPHO6) consisted of a 43 bp oligonucleotide which was complementary to a sequence encoding the amino acid sequence GCRC(I/V)E(L/I)D(C/V)W(D/N)G and which also included the 6 bp recognition sequence for the restriction endonuclease *Xba*I at the 5' end. PCR amplification was performed in a 100 µl reaction mixture containing 60 pmol each of the forward and reverse primers, 10 mM deoxynucleoside triphosphates, 2.5 mM MgCl₂, 10 mM Tris (pH 9.0 at 25 °C), 50 mM KCl, 0.1% (v/v) Triton X-100, 2.5 U *Taq* DNA polymerase and 500 ng *C. albicans* 132A total cellular DNA. PCR reactions were performed in a DNA thermal cycler (Perkin Elmer Cetus) with 35 cycles of 1 min at 94 °C, 2 min at 37 °C and 3 min at 72 °C. To complete the last cycle of amplification, the mixture was incubated for 10 min at 72 °C. Following amplification, 10 µl aliquots of each PCR mixture was subjected to agarose gel electrophoresis using 0.5 × TBE buffer through either 2% (w/v) low-EEO agarose (Sigma) or 2% (w/v) Seakem agarose (FMC) gels containing 0.5 µg ethidium bromide ml⁻¹ and the amplimers viewed on a UV transilluminator (UVP). Experiments were repeated on at least two occasions with separate preparations of target genomic DNA.

Cloning of PCR products. Individual PCR products were separately purified from Seakem agarose gels following electrophoresis by excising the appropriate portion of agarose into a sterile 1.5 ml Eppendorf microfuge tube containing 500 µl sterile distilled water followed by boiling for 5 min. Three microlitres of this extract were then used as a DNA template for further PCR experiments as before except that annealing temperatures of 42 °C and 45 °C were used instead of 37 °C. These higher annealing temperatures ensured that only single PCR products were subsequently amplified. These were purified immediately following amplification by extraction with an equal volume of phenol/chloroform (1:1, v/v) followed by precipitation with 0.3 M sodium acetate and 2 vols ice-cold 100% (v/v) ethanol. Aliquots of each purified PCR product were then co-digested with *Eco*RI and *Xba*I and cloned into *Eco*RI- and *Xba*I-cleaved vector plasmid pBluescript.

DNA sequencing. DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (1977) as described by Sambrook *et al.* (1989) using an automated Applied Biosystems 370A DNA sequencer and fluorescent-dye-labelled primers (FLASH kit, Genpak). Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST family of computer programs (Altschul *et al.*, 1990). Protein sequence analysis was performed using the programmes within the GCG Wisconsin package (Genetics Computer Group, 1994).

Purification of *C. albicans* 132A cellular DNA for library construction. High-molecular-mass total cellular DNA from *C. albicans* strain 132A was isolated using a modification of the method described by Sullivan *et al.* (1995) as follows. After zymolyase treatment, the resulting spheroplasts were harvested and washed once in TE buffer and resuspended in 1 ml of 25% (w/v) sucrose in 50 mM Tris/HCl, 1 mM EDTA at pH 8.0. This solution was incubated at 37 °C for 10 min and then transferred to ice followed by the addition of 2 mg proteinase K and then 400 µl 0.5 M EDTA, pH 8.0, and 250 µl 10% (w/v) sodium N-lauroyl sarcosinate. After thorough mixing, the spheroplast suspension was incubated on ice for 90 min, after which it was transferred to a shaking waterbath at 50 °C and incubated for 16 h. Following incubation, to each lysate was added 8 ml of a CsCl solution consisting of 69.9 g CsCl, 55.2 ml TE buffer and 50 µg PMSF ml⁻¹. This mixture was then transferred into 10 ml Quickseal ultra-clear cen-

trifuge tubes (Beckman) and centrifuged for 40 h at 160 000 *g* at 10 °C in a Beckman 70.1 Ti fixed-angle rotor using an L8-60M ultracentrifuge (Beckman). After centrifugation, DNA was collected from the tubes by side-puncture with an 18 gauge syringe needle (Microlance 2, Becton-Dickson). Caesium chloride was removed from the DNA by dialysis overnight (16 h) against 5 litres 1 × TE buffer at 4 °C using dialysis tubing (Sigma). DNA was recovered by ethanol precipitation, dried, resuspended in TE buffer at pH 8.0 and stored in aliquots at -20 °C.

Construction of a *C. albicans* 132A genomic DNA library. A lambda EMBL3 library was constructed by ligating *Sau*3A-generated partial digest products of *C. albicans* 132A chromosomal DNA greater than 10 kb in size with *Bam*HI-generated pre-prepared lambda bacteriophage replacement vector EMBL3 arms (Promega) followed by packaging *in vitro* into pre-prepared phage heads and tails (Promega) according to the manufacturer's instructions. Lambda EMBL3 has been shown previously to have a cloning capacity of 9–23 kb (Frischauf *et al.*, 1983). Following *in vitro* packaging, recombinant phage particles were propagated on the *E. coli* P2 lysogenic strain P2 392 as described by Sambrook *et al.* (1989). A recombinant lambda library containing 7.5×10^4 p.f.u. was obtained. The probability of a given sequence not being contained in the library was calculated as 2.4×10^{-17} , using the equation of Kurtz *et al.* (1990), estimating the *C. albicans* genome as approximately 29.4 Mb in size and estimating the mean insert size in the library to be approximately 15 kb.

Recombinant phages were propagated on *E. coli* LE392 to yield ~600–700 p.f.u. per plate for 10 × 90 mm Petri plates and were transferred from the plaques onto nitrocellulose membrane filters (Schleicher and Schuell) by overlaying the plaques with the filters, which were then screened by plaque hybridization (Sambrook *et al.*, 1989) using the α -³²P-labelled cloned amplicon contained in pDB001. The genomic DNA of a recombinant EMBL3 phage, termed P7, which hybridized strongly with the probe was purified as described by Sambrook *et al.* (1989). The cloned DNA insert of P7 (Fig. 1) was mapped with restriction endonucleases and specific fragments were subcloned into pBluescript by conventional methods (Sambrook *et al.*, 1989).

Pulsed-field gel electrophoresis (PFGE). Yeast chromosomes were prepared in agarose plugs as described by Vazquez *et al.* (1991) and were separated in 1.3 % (w/v) agarose gels using the CHEF-Mapper PFGE system (Bio-Rad). The electrophoresis buffer used was 0.5 × TBE and was maintained at 14 °C using buffer recycling through a Bio-Rad minichiller (model 1000). Gels were run for 22 h with an initial switch time of 60 s and a final switch time of 280 s, with a ramping factor of -2.379. Following electrophoresis, gels were stained with 0.5 µg ethidium bromide ml⁻¹ for 20 min, destained for between 20 and 90 min in distilled water and viewed on a UV transilluminator.

Preparation of cell-free protein extracts from *Candida* strains and isolates. Cell-free protein extracts from *Candida* strains and isolates were prepared by mechanical lysis using glass beads. Briefly, separate 50 ml volumes of YPD broth were inoculated with 1×10^5 cells ml⁻¹ of each strain or isolate, and placed in a shaking orbital incubator, set at 200 r.p.m., for 20 h at 37 °C, after which time the cultures had a final cell density of approximately 1×10^8 cells ml⁻¹. The inocula were prepared by diluting cells from 4 h YPD broth cultures in sterile saline to yield the required cell density. Cells were harvested by centrifugation at 5900 *g* (Sepatech Megafuge 1.0, Heraeus) for 10 min and washed once in sterile saline. Yeast cell pellets were resuspended in 1 ml *p*-nitrophenylphosphoryl-

choline (*p*-NPPC) assay buffer (60 %, w/v, sorbitol buffered with 0.25 M Tris/HCl, pH 7.2; Kurioka & Matsuda, 1976). This suspension was lysed using 1 g glass beads (0.45–0.5 mm diameter; Sigma) by vortexing in a Maximix Plus (Thermolyne) vortex using a cycle of 5 × 1 min with 1 min intermittent cooling on ice. The glass beads were then separated by centrifugation for 5 min at 1500 *g* and the resulting supernatant was further centrifuged at 10600 *g* in an Eppendorf centrifuge (model 5417C) for 5 min to yield a cell-free protein extract. The concentration of total protein (mg ml⁻¹) in each extract was determined using a kit based on the Bradford protein assay system (Bio-Rad) used according to the manufacturer's instructions.

Preparation of cell-free protein extracts from *E. coli* strains harbouring pBluescript and derivatives. The method used to prepare cell free homogenates from *E. coli* DH5α derivatives harbouring plasmids was essentially that described above for *Candida* strains and isolates, except that L broth (supplemented with 100 µg ampicillin ml⁻¹ to maintain plasmids) was used as growth medium and a lysis cycle of 3 × 1 min with 1 min with intermittent cooling on ice was used. Cell-free supernatants were generated and protein concentrations determined as described above.

Spectrophotometric assay of phospholipase C activity. *Candida* strains and isolates and *E. coli* DH5α derivatives harbouring plasmids pBluescript, pDB008, pDB009, pDB010, pDB016 or pDB017 were assayed for PLC activity using the phospholipase-C-specific substrate *p*-NPPC by a modification of the method described by Kurioka & Matsuda (1976). Briefly, 90 µl freshly prepared protein extract from each isolate was assayed in 96-well round-bottomed microtitre plates (Corning) by the addition of 10 µl 200 mM *p*-NPPC (20 mM final concn) in 60 % (w/v) sorbitol buffered with 0.25 M Tris/HCl (pH 7.2), and incubated at 37 °C for 20 h. The rate of *p*-NPPC hydrolysis by PLC was determined by measuring free *p*-nitrophenol at 405 nm using an ELISA plate reader (Spectra I; SLT-Labinstruments). For each series of test experiments, standard concentrations of PLC (type IX, from *Clostridium perfringens*; 6.6 units of activity per mg of solid; Sigma) with known units of activity in µg ml⁻¹ were assayed in a reaction mixture containing 20 mM *p*-NPPC, 60 % (w/v) sorbitol buffered with 0.25 M Tris/HCl (pH 7.2) at 37 °C for 20 h to generate a standard curve of PLC activity for each experiment. The activity units of PLC (based on concentration) for each isolate tested were calculated from the slope of the standard curve generated in the same experiment. The activity of PLC was expressed as units per mg total protein in cell-free extract for each isolate tested. One unit of activity was defined as the amount of enzyme that will liberate 1.0 µmol *p*-nitrophenol from *p*-NPPC min⁻¹ at 37 °C, pH 7.2.

RNA isolation, reverse transcription and PCR. Total cellular RNA was isolated from 7 h and 24 h *C. albicans* 132A blastospore cultures grown in YPD broth medium at 37 °C and from hyphal cultures grown in Yeast Nitrogen-base Proline medium at 37 °C (NYP; Gallagher *et al.*, 1992) using the procedure of Hube *et al.* (1994). Total cellular RNA was also isolated from 7 h YPD broth cultures of the oral isolates *C. dubliniensis* CD48-I, *C. tropicalis* 709A, *C. glabrata* 803A and *C. parapsilosis* 701A using the same procedure. Total RNA was reverse transcribed in a reaction volume of 20 µl containing 1 mM each of dATP, dGTP, dCTP and dTTP, 0.5 µg (µg RNA)⁻¹ of oligo(dT)₁₅ as primer, a buffer consisting of 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 5 mM MgCl₂ and 0.1 % (v/v) Triton X-100 and 15 units of avian myeloblastosis virus reverse transcriptase, in the presence of rRNasin ribonuclease inhibitor (RT-PCR kit, Promega). The reaction

Table 1. PCR primers used in this study

Primer name	Sequence*	Length (bp)	RE site†
CAPHO5	5'-TCGGAATTCTSYCAYAAAYACKTAYCT-3'	26	<i>EcoRI</i>
CAPHO6	5'-GCTCTAGACCRYYCARMWRTCRAYYTCKAYACAGCGRCAKCC-3'	43	<i>XbaI</i>
XDOM F	5'-ACTGGTTCGACAAGTTGCTGG-3'	20	<i>Sall</i>
XDOM R	5'-AGCAAAGCTTATTGGTTTAG-3'	20	<i>HindIII</i>
PLCS2	5'-TCGGAATTCTCGAAAAGTAAAGACGG-3'	26	<i>EcoRI</i>
PLCE	5'-GAAGTACTAGTTGTTATCTTCCCCATG-3'	24	<i>SpeI</i>
ACT F	5'-CGGAATTCCGCTTCTTCTCAATCTTCTGCC-3'	30	<i>EcoRI</i>
ACT R	5'-CGGAATTCCGAATGGATGGACCAGATTCGTCG-3'	32	<i>EcoRI</i>

*R = A or G; Y = C or T; K = G or T; M = A or C; S = G or C; W = A or T.

†Restriction endonuclease recognition sequence included within the primer sequence (underlined).

was carried out at 42 °C for 60 min. Approximately equal amounts of (50 ng) RNase-free DNase-treated total cellular RNA was added for each sample as determined spectrophotometrically (A_{260}) and visually following electrophoresis in 1.5% (w/v) agarose gels containing 6% (v/v) formaldehyde as described by Hube *et al.* (1994). PCR reactions were performed in a Perkin Elmer Cetus DNA thermal cycler. The reactions were essentially those described for DNA PCR (see above) except that reaction mixtures contained 20 pmol of each primer and 8 µl of each cDNA sample was added as template. The primers used were XDOM F and XDOM R (Table 1). PCR conditions were 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a further 10 min at 72 °C. Reaction products were analysed by electrophoresis through 2% (w/v) agarose gels. In addition to a PCR negative control to which no template was added, other negative controls included reactions in which DNase-treated RNA that had not been reverse transcribed was used as a template for each RNA preparation tested. These controls were used to ensure that RNA preparations used for cDNA synthesis were not contaminated with cellular DNA. Genomic DNA from *C. albicans* 132A was used as a positive PCR control in each PCR experiment. Two primers, ACT F and ACT R (Table 1), were designed to amplify a 440 bp product from within the *C. albicans* actin gene (Losberger & Ernst, 1989) and these were used with each cDNA sample tested to act as a positive control for cDNA synthesis and subsequent PCR.

Northern analysis. For Northern blots, total RNA was extracted and electrophoresed in 1.5% (w/v) agarose gels containing 6% (v/v) formaldehyde as described by Hube *et al.* (1994). Following electrophoresis, RNA was transferred to MagnaGraph nylon membrane filters (MSI) by capillary action and the RNA was bound to the membranes by baking at 80 °C for 30 min and UV cross-linking as described by Hube *et al.* (1994). Probe DNA was labelled as described above for Southern analysis. Membrane filters containing bound RNA were hybridized at 42 °C by the method of Hube *et al.* (1994) using a rotary hybridization oven (Hybaid). Detection of the translation elongation factor 3 gene (*TEF3*) mRNA transcript (Colthurst *et al.*, 1992), which is constitutively expressed in *C. albicans* yeast and hyphal forms (Swoboda *et al.*, 1994), was used as a positive control in all Northern experiments using an 0.7 kb internal fragment of the *TEF3* gene as described by Hube *et al.* (1994) as a probe. Membranes were stripped of bound probe for subsequent rehybridization by immersion in boiling water for 10 min.

Cloning the entire *CAPLC1* gene. To obtain a clone containing the entire *CAPLC1* gene, PCR primers were designed to amplify the entire open reading frame of 3297 bp and the transcriptional control sequences (an additional 474 bp). PCR amplifications were performed in a Perkin Elmer Cetus DNA thermal cycler in a final reaction volume of 100 µl which contained 300 nM of each primer, 200 µM deoxynucleotide triphosphates, 2.25 mM $MgCl_2$, 50 mM Tris/HCl (pH 9.2 at 25 °C), 14 mM $(NH_4)_2SO_4$, 2% (v/v) dimethyl sulfoxide, 0.1% (v/v) Tween 20, 2.6 U *Taq* and *Pwo* DNA polymerase enzyme mix (Expand High Fidelity PCR system, Boehringer Mannheim) and 500 ng *C. albicans* 132A total cellular DNA. The primers used were PLCS2 and PLCE (Table 1). After an initial denaturation step of 94 °C for 2 min, PCR conditions were 10 cycles of 94 °C for 10 s, 45 °C for 30 s and 68 °C for 4 min followed by 15 cycles of 94 °C for 10 s, 45 °C for 30 s and 68 °C for 4 min with an extension of 20 s for each cycle at the elongation temperature, followed by a prolonged elongation step of 7 min at 68 °C. Reaction products were analysed by electrophoresis through 0.8% (v/v) agarose gels. PCR products were purified immediately after amplification by extraction with an equal volume of phenol/chloroform (1:1, v/v) followed by precipitation with 0.3 M sodium acetate and 2 vols ice-cold 100% (v/v) ethanol. Aliquots of each purified PCR product were then co-digested with *EcoRI* and *SpeI* and cloned into *EcoRI*- and *SpeI*-cleaved vector plasmid pBluescript.

RESULTS AND DISCUSSION

Production of phospholipase C by *C. albicans* 132A

PI-PLC activity in cell-free extracts of *C. albicans* 132A was determined spectrophotometrically using the PI-PLC-specific chromogenic substrate *p*-NPPC. Extracts of *C. albicans* 132A from cultures grown in the yeast phase in YPD broth for 20 h yielded a PLC activity of $15.1 \pm 1.8 \times 10^{-2}$ units (mg protein)⁻¹ (mean $\pm$ SD, $n = 7$). In contrast, extracts from cultures grown in the hyphal phase in NYP broth for 20 h yielded a PLC activity of $39.0 \pm 1.7 \times 10^{-2}$ units (mg protein)⁻¹ (2.6-fold increased activity). Similar results were obtained in separate experiments. The significance of the higher PLC activity in the hyphal phase compared to the yeast phase is presently unknown but could reflect relative rates of membrane synthesis in the two phases. These

Table 2. Predicted amino acid consensus regions within the X domain encoded by previously characterized phospholipase C genes used for oligonucleotide primer design

Species	Gene name	GenBank accession no.	Consensus amino acid sequences used for primer design	
			Forward primer	Reverse primer
<i>Saccharomyces cerevisiae</i>	SCPLC	L13036	SSHNTYL	GCRCVEIDIWDG
	SCPLC1	D12738	SSHNTYL	GCRCVEIDIWDG
<i>Dictyostelium discoideum</i>	DDPHOSPHC	M95783	SSHNTYL	GCKCVELDVWDG
<i>Drosophila melanogaster</i>	DMPLC21A	M60452	SSHNTYL	GCRCVELDFWNG
	DMPLC21B	M60453	SSHNTYL	GCRCVELDFWNG
	DMNORPA	J03138	SSHNTYL	GCRCVELDCWNG
<i>Xenopus laevis</i>	XLPHSLPC	L20816	SSHNTYL	GCRCIELDCWKG
<i>Rattus norvegicus</i>	RSPLC3A	M20637	SSHNTYL	GCRCLELDCWDG
	RNBETA4AA	L15556	SSHNTYL	GCRCIELDCWDG
	RNPLPIV	J05155	SSHNTYL	GCRCIELDCWDG
	RRPLCA	J03806	SSHNTYL	GCRCIELDCWDG
<i>Bos taurus</i>	BTPHOSA	L13935	SSHNTYL	GCRCVELDCWDG
	BTPHOSB	L13936	SSHNTYL	GCRCVELDCWDG
	BTPHOSC	L13937	SSHNTYL	GCRCVELDCWDG
	BTPHOSD	L13938	SSHNTYL	GCRCVELDCWDG
	BTPILC	J03137	SSHNTYL	GCRCVELDCWKG
	BTPLC3A	M20638	SSHNTYL	GCRCLELDCWDG
<i>Homo sapiens</i>	HSPLC	X14034	SSHNTYL	GCRCIELDCWDG
	HSPLCA	M34667	SSHNTYL	GCRCIELDCWDG
	HSPLPC	Z16411	SSHNTYL	GCRCVELDVWKG
	HSPLC01	M37238	SSHNTYL	GCRCIELDCWDG
	HSPLCB2A	M95678	SSHNTYL	GCRCVELDCWKG

results demonstrated unequivocally that *C. albicans* 132A expressed PLC activity.

Identification of a *C. albicans* phospholipase C gene

In an attempt to identify a *C. albicans* PLC determinant using PCR, a pair of degenerate oligonucleotide primers, termed CAPHO5 and CAPHO6 respectively, were designed (Table 1), which were complementary to sequences encoding two non-contiguous conserved amino acid regions from within the X domains of 22 previously characterized phospholipase C (PLC) genes from seven separate eukaryotic species (Table 2). The predicted sizes of amplicons which would be obtained with target DNA from these genes (Table 2) using these primers would be approximately 130 bp in each case. Ten PCR products were obtained with target genomic DNA from *C. albicans* 132A, ranging in size from 100 to 800 bp, four of which were >350 bp. Because the predicted size of amplicons obtainable with the PLC genes listed in Table 2 was much smaller, the four larger *C. albicans* 132A amplicons were not studied further. Each of the six smaller amplicons was purified and cloned separately into pBluescript, and their nucleotide sequences were determined. The sequence of one amplicon of 130 bp cloned in the recombinant plasmid pDB001 was found to be highly homologous (65 % sequence identity) to the corresponding nucleotide

sequence encoding the X domain of the PLC gene of the fission yeast *Schiz. pombe* (67 % predicted amino acid identity, 91 % similarity; Fankhauser *et al.*, 1995). Sixty-nine basepairs of the 130 bp amplicon cloned in pDB001 consisted of primer sequences used for amplification; the deduced amino acid sequence of the remaining 61 bp was 68 % identical (89 % similar) to residues of the X domain encoded by the *Schiz. pombe* PLC gene. These data indicated that pDB001 was likely to encode part of a *C. albicans* 132A PLC determinant and it was subsequently used as a probe to screen a *C. albicans* 132A genomic DNA library. None of the remaining five cloned amplicons showed any significant sequence homology to other PLC genes in the GenBank database. The sequence of the amplicon cloned in pDB001 is indicated in Fig. 2.

Isolation and physical mapping of a *C. albicans* phospholipase C gene

Plaques from a *C. albicans* 132A genomic DNA library constructed in the lambda vector EMBL3 were hybridized with the α -³²P-labelled insert from pDB001 and hybridization signals were obtained which corresponded to seven individual plaques from a total of approximately 7000 screened. The recombinant phage from the plaque yielding the strongest signal was chosen for further study and the phage termed P7. Phage P7

Fig. 1. (a) Restriction map of the *C. albicans* 132A genomic DNA cloned in recombinant phage P7, which is represented by the black rectangular box. The dashed lines at both ends of the rectangular box represent phage EMBL3 sequences. The shaded box to the left represents the location of a 0.5 kb *AvaI* fragment of EMBL3 DNA which was subcloned along with the 4.5 kb *AvaI* fragment of P7 insert DNA in recombinant plasmid pDB005. The small hatched area within the black rectangular box represents the location of sequences corresponding to the insert DNA of pDB001. The thin black lines in the lower part of the figure represent specific fragments of P7 insert DNA that were subcloned in vector plasmid pBluescript. The names of the recombinant plasmids are shown on the right. (b) The thin black lines represent specific fragments of pDB005 that were subcloned from P7 insert DNA in pBluescript. The names of the plasmids are shown on the right. (c) Restriction map of recombinant plasmid pDB017 encoding the entire *C. albicans* CAPLC1 gene, which is represented by a white rectangular box, on a 3.7 kb *EcoRI*–*SpeI* fragment amplified by PCR from *C. albicans* 132A genomic DNA and cloned in pBluescript. The arrowed line at the top represents the position and shows the direction of transcription of the ORF encoding the CAPLC1 gene. The hatched boxes represent sequences encoding the X and Y domains of the predicted translation product encoded by CAPLC1. Restriction endonuclease cleavage sites are abbreviated as follows: S, *SalI*; A, *AvaI*; C, *ClaI*; E, *EcoRI*; EV, *EcoRV*; H, *HindIII*; Sa, *Sau3A*; Sp, *SpeI*; B, *BamHI*; BX, *BstXI*; Bg, *BglII*; X, *XbaI*.

harboured a cloned DNA insert of approximately 18 kb which contained a single internal *SalI* restriction site (Fig. 1). The cloned amplicon in pDB001 also contained a single internal *SalI* site, which indicated that the region of shared DNA homology with the DNA of P7 spanned the internal *SalI* site (Fig. 1). Furthermore, pDB001 insert DNA hybridized with each of the 4.0 kb *SalI*, the 4.5 kb *SalI*–*XbaI* and the 4.5 kb *AvaI* fragments of P7 following subcloning in pBluescript to yield plasmids pDB003, pDB004 and pDB005, respectively (Fig. 1). This evidence, together with the homology shared between the cloned amplicon DNA in pDB001 and the *Schiz. pombe* PLC determinant, suggested that the region of P7 DNA spanning the internal *SalI* site very probably encoded a PLC determinant from *C. albicans* 132A. To investigate this possibility further, the 3.0 kb *AvaI*–*SpeI* fragment of pDB005 was subcloned into plasmid pBluescript to yield pDB008. The entire nucleotide sequence of the 3.0 kb *AvaI*–*SpeI* insert of pDB008 was determined; part of it is shown in Fig. 2.

While subcloning fragments of the insert DNA from phage P7 into pBluescript, recombinant plasmids harbouring only the 4.5 kb *AvaI* fragment which hybridized with pDB001 were never recovered. In two separate experiments, *AvaI* fragments of P7 were ligated into *AvaI*-digested pBluescript; following transformation into *E. coli* DH5 α 169 transformants harbouring recombinant plasmids were screened, 19 of which were found to contain the 4.5 kb *AvaI* fragment in conjunction with an additional 0.5 kb *AvaI* fragment as in pDB005 (Fig. 1). None of the transformants harboured the 4.5 kb *AvaI* fragment alone. Attempts to recover pDB005 derivatives which had lost the 0.5 kb *AvaI* fragment following digestion with *AvaI* followed by ligation and transformation into DH5 α -were unsuccessful, despite repeated attempts. Furthermore, recombinant derivatives harbouring the 3.0 kb *AvaI*–*SpeI* fragment of pDB005 subcloned in pBluescript as in pDB008 also contained this 0.5 kb *AvaI* fragment (Fig. 1). Nucleotide sequence analysis of this 0.5 kb *AvaI*

Fig. 2. Nucleotide sequence and deduced amino acid sequences of the region of the *CAPLC1* gene encoding the X and Y domains. Nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon. Amino acid sequences are numbered from the initial methionine. Amino acid residues of the CAPLC1 X domain (residues 569–727) and Y domain (residues 793–913) are underlined. Conserved residues thought to be important for binding IP₃ and Ca²⁺ in the X and Y domains (active site) in previously characterized PLC genes are highlighted. Nucleotide sequences corresponding to the amplicon cloned in pDB001 are indicated by dotted underlining (nucleotide co-ordinates 1728–1845). Two potential polyadenylation signal sequences (starting at nucleotides 3445 and 3480) are doubly underlined. The positions of unique restriction endonuclease cleavage sites are shown.

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The *CAPLC1* ORF has the capacity to encode a protein of 1099 amino acids with 16 predicted glycosylation sites, all asparagine residues, with a predicted molecular mass of 124.6 kDa and a pI of 8.16, which is larger in size compared to δ -form PI-PLCs of other yeasts, including that of *Sacch. cerevisiae* (869 amino acids; Yoko-o *et al.*, 1993) and *Schiz. pombe* (899 amino acids; Fankhauser *et al.*, 1995). Comparison of the deduced amino acid sequence of the *CAPLC1* protein with the predicted amino acid sequences of δ -form PLCs encoded by the eight most similar *PLC* genes representative of different

Fig. 3. Comparison of the primary structure of the predicted translation product of the CAPLC1 gene and the corresponding translation products of the eight most similar PLC genes representative of different eukaryotic species encoding phospholipase C δ -1 isoforms in the GenBank database. The percentage overall amino acid identity of CAPLC1 to each of the other phospholipases is shown under each phospholipase name and the percentage similarity is shown in parentheses. The X and Y domains of each phospholipase are shown as hatched boxes. The percentage amino acid identity between the CAPLC1 X and Y domains, respectively, and the corresponding regions of the other phospholipases are indicated beneath each phospholipase and the percentage similarity is shown in parentheses. The phospholipases compared are as follows: CAPLC1, *C. albicans* (this study); SCPLC, *Sacch. cerevisiae*; SPPLC, *Schiz. pombe*; DDPHOSPHC, *Dictyostelium discoideum*; CGPLCD1, *Cricetulus griseus*; RSPLC3A, *Rattus norvegicus*; HSU09117, *Homo sapiens*; OCPLCMR, *Oryctolagus cuniculus*; BTPLC3A, *Bos taurus*. The dashed line at the N-termini of CGPLCD1, OCPLCMR and BTPLC3A indicates that the complete N-terminal sequence was not available from the GenBank database.

eukaryotic species in the GenBank database, including *Schiz. pombe* (Fankhauser *et al.*, 1995), *Sacch. cerevisiae* (Yoko-o *et al.*, 1993), *Dictyostelium discoideum* (Drayer & van Haastert, 1992), *Bos taurus* (Suh *et al.*, 1988), *Rattus norvegicus* (Suh *et al.*, 1988), *Cricetulus griseus* (Leonis & Silbert, 1996), *Oryctolagus cuniculus* (Milting *et al.*, 1996) and *Homo sapiens* (Cheng *et al.*, 1995) shows more than 19% overall amino acid identity (>32% similarity) over the entire protein (Fig. 3). The predicted CAPLC1 protein exhibits many of the features common to other PLCs, especially with δ -form isoenzymes, including amino acid regions which correspond to the X and Y domains that are important for catalytic activity in other PLCs (Rhee *et al.*, 1989; Rhee & Choi, 1992; Ellis *et al.*, 1993; Yagisawa *et al.*, 1994; Cheng *et al.*, 1995; Essen *et al.*, 1996). The N-terminal part of the CAPLC1 protein preceding the X domain shows <20% sequence identity with the predicted amino acid sequences of the eight most similar PLC genes representative of different eukaryotic species and is significantly larger in size in each case, at 568 amino acids in length (the N-termini of the eight most similar PLC genes representative of different eukaryotic species range from between 239 and 445 amino acids in length). Surprisingly, the N-terminal region of the CAPLC1 protein did not contain a recognizable EF-hand domain (putative Ca^{2+} -binding site) using the Motifs program of the GCG Wisconsin software package (Genetics Computer Group, 1994). Furthermore, no sequence within the CAPLC1 protein shared any significant similarity with 45 known EF-hand proteins (R. Kretsinger, personal communication). However, as three-dimensional

structure is more conserved than amino acid sequence, an EF-hand domain or else other sequences which perform the same role may still be present within CAPLC1. This finding was unique to the predicted CAPLC1 protein, as DNA sequences of all previously characterized eukaryotic PLC genes, including those from yeasts and slime moulds, encoded a relatively conserved 29-residue EF-hand domain sequence (Bairoch & Cox, 1990; Essen *et al.*, 1996). It has long been known that mammalian PLC δ 1 enzymes have an absolute requirement for Ca^{2+} for activity and the EF-hand domain is thought to be essential for activity (Mitchell, 1992; Nishizuka, 1992; Berridge, 1993). This has been confirmed for rat brain PLC δ 1, as deletion of approximately one half of the EF-hand domain completely abolished enzyme activity (Nakashima *et al.*, 1995). The precise role of the EF-hand domain is unclear but one possibility is that it may be important in maintaining the C-terminal region in the appropriate three dimensional configuration (Essen *et al.*, 1996).

The region of CAPLC1 corresponding to the X domain is 159 amino acids in size, which is greater than the predicted sizes for this domain of between 137 and 141 amino acids for the other eight most similar PLC genes representative of different eukaryotic species in the GenBank database. This variation in size is due to a stretch of 18–19 additional amino acids located within the CAPLC1 X domain which do not align with any residues within the corresponding X domains of all other PLCs (19 residues for yeast PLCs and 18 residues for PLCs from higher eukaryotes) in the EMBL database

CAPLC1	569	PLNEYFISSSHNTYLTGRQVAGDSSVEGYIRTLQRCRCVEIDIWNGDSNTMTTTVIGTKDDDDKNEYEPVNHGRITFTK	
SCPLC	384	PLNHFIASSHNTYLLGKQIAETPSVEGYIQVLQGGRCRCVEIDIWDGENG-----PVVCHG-FLTS	
SPPLC	445	PLNEYLISSSHNTYLLGKQFGGESSIEGYIRSLQRCCKCIEIDCWDPNG-----PVVCHGHTFTS	
DDPHOSPHC	326	PLSYFINSSSHNTYLSGHQLKGLSTSEMYTNTLRQCKCVELDVWDGNDGD-----PIIFHGNTLTS	
CGPLCD1S3	289	PLSHYLVSSSHNTYLLDQLTGPSSTEAYIRALCKGRCRCLELDCWDGPNQE-----PIIYHGYTFTS	
RSPLC3A	300	PLSHYLVSSSHNTYLLDQLTGPSSTEAYIRALCKGRCRCLELDCWDGPNQE-----PIIYHGYTFTS	
HSU09117	301	PLSHYLVSSSHNTYLLDQLAGPSSTEAYIRALCKGRCRCLELDCWDGPNQE-----PIIYHGYTFTS	
OCPLCMR	289	PLSHYLVSSSHNTYLLDQLTGPSSTEAYIRALCKGRCRCLELDCWDGPNQE-----PVIYHGYTFTS	
BTPLC3A	239	PLSHYLVSSSHNTYLLDQLTGPSSTEAYIRALCKGRCRCLELDCWDGPNQE-----PIIYHGYTFTS	
		* * * . * * * * * * * . * * * * . * * * * . * * * * . * * * * . * * * * . * * * * .	
CAPLC1	649	PISFANVIRAIAKFAFVSPWPLILSLEIHCSSPECQIKVNVILKDIILGENMIIAPIDIDSILPSPAECLKHKFIKVKK	727
SCPLC	444	AIPLKTVIRVIKKYAFITSPYPLIISLEINCNDQKLASLIMREVLAEQLYFVGTRTDK--LSPRELKHKILLKSKK	521
SPPLC	506	MIKFNDVIDAIRKYAFVSPYPLFISLEIHCCPDQQRQMVSYMKAQFDTLVMPVTANESVLPSPEDLLNKILLKVKC	585
DDPHOSPHC	386	QIKFHVCEITIKARGFETSPYPVILSLEVHCSVPQQIMMANHMEIFG-EMLPPLPEGTKEPLTDSLKYKILLKG--	464
CGPLCD1S3	351	KILFYDLVRAIRDYAFKASPYPVILSLENHCSLEQQQVMARHLKAILGPMILLDQPLDGVVTMSLPSEQLKGGKILLKGGK	430
RSPLC3A	362	KILFCDVLRIRDYAFKASPYPVILSLENHCSLEQQQVMARHLRAILGPILLDQPLDGVVTMSLPSEQLKGGKILLKGGK	441
HSU09117	363	KILFCDVLRIRDYAFKASPYPVILSLENHCSLEQQQVMARHLRAILGPILLDQPLDGVVTMSLPSEQLKGGKILLKGGK	442
OCPLCMR	351	KILFCDALRAIRDYAFKASPYPVILSLENHCSLEQQQVMARHLRTLLGPMILLDRPLDGVVTSSLPSEQLKGGKILLKGGK	430
BTPLC3A	301	KILFCDVLRIRDYAFKASPYPVILSLENHCSLEQQQVMARHLRTLLGPMILLDRPLDGVVTSSLPSEQLKGGKILLKGGK	380
		* * * . * * * * * * * . * * * * . * * * * . * * * * . * * * * . * * * * . * * * * .	

Fig. 4. Alignment of the predicted amino acid sequence of the X domain of the *CAPLC1* gene and the corresponding predicted amino acid sequences of the X domains of the eight most similar PLC genes representative of different eukaryotic species in the GenBank database. The X domain encoded by *CAPLC1* contains a novel region consisting of 18–19 amino acid residues, a corresponding region for which is absent from the X domains of the other phospholipases. Amino acid sequences were aligned using the CLUSTAL sequence alignment computer program (Higgins & Sharp, 1988). The phospholipase residues aligned were as follows: *CAPLC1*, *C. albicans* (this study); *SCPLC*, *Sacch. cerevisiae*; *SPPLC*, *Schiz. pombe*; *DDPHOSPHC*, *Dictyostelium discoideum*; *CGPLCD1*, *Cricetulus griseus*; *RSPLC3A*, *Rattus norvegicus*; *HSU09117*, *Homo sapiens*; *OCPLCMR*, *Oryctolagus cuniculus*; *BTPLC3A*, *Bos taurus*. Asterisks indicate identical residues and dots represent similar residues. Dashes indicate gaps created to obtain alignment.

(Fig. 4). These residues are highly hydrophilic, have a high surface probability (Emeni *et al.*, 1985), have a high chain flexibility (Karplus & Schulz, 1985) and have a high Jameson–Wolf antigenicity index (Wolf *et al.*, 1987) as determined by the PeptideStructure computer program (Genetics Computer Group, 1994). However, despite these additional amino acid residues the X domain of *CAPLC1* exhibits more than 42% amino acid identity (>59% similarity) to the corresponding X domains of the other eight PLCs indicated in Fig. 4. The *CAPLC1* X domain also contains two conserved histidine residues (at positions 579 and 642) which have been shown to be essential for enzyme activity in previously characterized PLCs (Fig. 2). The X domain also contains conserved asparagine, glutamine, aspartic acid and glutamine residues at positions 580, 609, 611 and 676, respectively, which have been shown to be essential for the binding of calcium in other PLCs (Fig. 2; Essen *et al.*, 1996). The region of the *CAPLC1* protein between the X and Y domains is 65 amino acids in length, which is within the range of 48–72 amino acids for the corresponding region of the other PLCs. This region also contains a stretch of 13 threonine residues which do not correspond with any residues within the corresponding regions of the other PLCs.

The region of *CAPLC1* corresponding to the Y domain is 121 amino acids in size, which is similar to the predicted size for this domain of 120 amino acids for the other eight PLC genes in the EMBL database (Rhee *et al.*, 1989; Drayer & van Haastert, 1992). This region exhibited >33% identity (>47% similarity) with the

Y domains of the eight most similar PLCs representative of different eukaryotic species in the GenBank database, and contained three residues (a serine at position 823, an arginine at position 852 and a tyrosine at position 854) which have been shown to be conserved in previously characterized PI-PLCs, and which are considered important in determining substrate specificity and for catalysis (Essen *et al.*, 1996; Fig. 2). Finally, the C-terminal part of δ -form PLC proteins after the Y domain can vary from 147 to 159 amino acids. The C-terminal part of the *CAPLC1* is 187 amino acids in size and is <26% homologous (<43% similar) to the C termini of the other PLC proteins examined.

Phenotypic expression of *CAPLC1* in *E. coli*

To investigate whether expression of the *CAPLC1* gene could be detected in *E. coli*, protein extracts from DH5 α containing either pDB017 or pDB008 or subclones derived from it were tested for PLC activity using the PLC-specific chromogenic substrate *p*-NPPC. A previous study has reported the expression of a trypanosomal PLC gene in *E. coli* (Mensa-Wilmot & Englund, 1992). Plasmid pDB017 contained the entire *CAPLC1* gene, including putative transcriptional control sequences, cloned on a 3.7 kb fragment amplified from *C. albicans* 132A template DNA using the primer set PLCS2 and PLCE (Table 1). Extracts from DH5 α harbouring pDB017 failed to exhibit levels of PLC activity above those detected with extracts of DH5 α harbouring pBluescript only. Because the cloned DNA within pDB017 was obtained by PCR amplification, it is

possible that misincorporation of single nucleotides could have occurred, leading to premature termination of transcription even though the proofreading polymerase *Pwo* was included with *Taq* polymerase during amplification. Similar results were obtained when five 3.7 kb amplimers, recovered in separate experiments with *C. albicans* 132A target DNA, were cloned in pBluescript and DH5 α extracts tested for PLC activity. It is also possible that the *CAPLC1* putative transcriptional control sequences contained in pDB017 (promoter and ribosome-binding site) do not function efficiently in *E. coli*.

Extracts from DH5 α harbouring pDB008 consistently (seven separate experiments) yielded an approximately twofold higher (mean 2.03 ± 0.35 higher) level of activity with the *p*-NPPC substrate compared to DH5 α harbouring pBluescript only. Furthermore, extracts from DH5 α harbouring pDB016 (Fig. 1) also consistently yielded higher levels of activity, although lower than those obtained with pDB008, compared to extracts from DH5 α harbouring pBluescript only or pDB010 (Fig. 1). In a typical experiment the activity in units (mg protein)⁻¹ detected in extracts from DH5 α -harbouring (i) pDB017 was 11.7×10^{-2} , (ii) pDB008 was 28.1×10^{-2} , (iii) pBluescript was 15.3×10^{-2} , (iv) pDB016 was 23.0×10^{-2} , (v) pDB009 was 19.8×10^{-2} and (vi) pDB010 was 17.6×10^{-2} . Similar results were obtained regardless of whether IPTG was present or absent from the growth medium, suggesting that the PLC activity expressed from the *CAPLC1*-encoding plasmids was not under control of the inducible *lac* promoter of pBluescript. Analysis of the *CAPLC1* sequences in pDB017, pDB016, pDB010 and pDB009 revealed that the cloned fragments were not in the correct reading frame to be expressed as fusion proteins with β -galactosidase.

The above expression studies indicated that the truncated *CAPLC1* gene (82%) encoded by pDB008 was expressed in *E. coli* at a low level. Furthermore, recombinant plasmid pDB016, which encoded a more extensively truncated *CAPLC1* gene (72%; Fig. 1), but which retained sequences encoding the X and Y domains of the *CAPLC1* protein, expressed residual PLC activity in *E. coli*. These results suggest that the *CAPLC1* N-terminus is not essential for PLC catalytic activity. Previous studies have shown that the N-terminal 135 amino acids of rat brain PLC δ 1 and the N-terminal 60 amino acids of bovine brain PLC δ 1 were not required for activity (catalysis) but were essential for substrate binding (Ellis *et al.*, 1993; Yagisawa *et al.*, 1994; Cifuentes *et al.*, 1993). It is likely that the PLC activity detected in *E. coli* DH5 α derivatives harbouring pDB008 or pDB016 was due to expression of a truncated *CAPLC1* protein, containing a functional catalytic domain in each case. The increased PLC activity detected in extracts of *E. coli* harbouring pDB008 compared to pDB016 could be due to translation of the truncated *CAPLC1* gene in pDB008 and the 0.5 kb *Ava*I EMBL3 sequence located 5' to these sequences as a fusion protein (see Fig. 1). The 0.5 kb *Ava*I fragment within pDB008 contained an internal TATA sequence and

encoded an in-frame methionine putative translational start site 18 bp 5' to the *Ava*I site within *CAPLC1* (Fig. 1). These results, together with similarities in the sequence homology of *CAPLC1* and in the structural organization of the deduced *CAPLC1* protein in comparison to other previously characterized PLC genes, provided convincing evidence that *CAPLC1* encodes a functional PLC determinant.

Plasmid pDB008, encoding 82% of the *CAPLC1* gene, had an adverse effect on the growth of *E. coli* DH5 α , which may have been due to expression of PLC activity. In eleven separate experiments the final cell density of 20 h L-broth cultures of DH5 α harbouring pDB008 was approximately half that obtained with DH5 α harbouring pBluescript only under the same experimental conditions. In seven separate experiments the protein concentration (mg ml⁻¹) in cell-free extracts from 20 h cultures of DH5 α harbouring pDB008 was consistently more than twofold lower (mean 2.36 ± 0.5 fold lower) compared to extracts from cells harbouring pBluescript only. In a typical experiment the protein concentration determined from cell extracts of DH5 α harbouring pDB008 was 2.13 mg ml⁻¹ compared to 5.02 mg ml⁻¹ for DH5 α harbouring pBluescript only. No significant difference was observed in the corresponding cell densities or protein concentrations of cell extracts of DH5 α harbouring pDB017, pDB016, pDB009, pDB010, pBluescript or pBluescript derivatives containing the 0.5 kb *Ava*I fragment of EMBL3 obtained following blunt-end cloning.

Extracts of *E. coli* DH5 α harbouring only pBluescript exhibited levels of PLC activity [$15 \pm 0.4 \times 10^{-2}$ units (mg protein)⁻¹] (mean $\pm$ SD, $n = 7$) which were similar to those detected with extracts of *C. albicans* 132A [$15.1 \pm 1.8 \times 10^{-2}$ units (mg protein)⁻¹]. The levels of PLC activity detected in the former extracts were higher than but of the same order of magnitude as the sum of PLC activities previously reported in three membrane fractions of *E. coli* strain B using a radiometric assay (Bayer & Bayer, 1985).

Expression of *CAPLC1* in *C. albicans*

To detect expression of the *CAPLC1* gene in *C. albicans* and to determine whether the sequences encoding the novel stretch of 19 amino acids in the X domain of the deduced translation product were expressed, a pair of primers termed XDOME and XDOME', respectively (Table 1), which were complementary to sequences flanking this region were made. These primers were designed to amplify a 210 bp amplicon encoding a 202 bp region of *CAPLC1* DNA (the remaining 8 bp consisted of primer clamp sequences and restriction sites; Table 1) corresponding to nucleotides 1750–1959 as shown in Fig. 2. These primers yielded a single amplicon of 210 bp following PCR amplification with target genomic DNA from *C. albicans* 132A. If the sequences encoding the novel stretch of 18–19 amino acids in the *CAPLC1* X domain were expressed, a product of 210 bp would be expected when cDNA from

Fig. 5. Agarose gels (2% w/v) containing ethidium-bromide-stained PCR products obtained following amplification using either total genomic DNA as template, or cDNA which had been synthesized following reverse transcription of DNA-free total cellular RNA as template, with the ACT primer set (upper panel in each case) and the XDOM primer set (lower panel in each case). All of the amplimers shown in the upper panels hybridized with the *C. albicans* ACT1 gene cloned in p1002 (Mason *et al.*, 1987). CAPLC1-homologous amplimers in the lower panels were identified by hybridization with the CAPLC1-encoding insert of pDB017 (Fig. 1). (a) The PCR products shown in the lanes (both upper and lower panels) were amplified from template DNA or cDNA from *C. albicans* 132A or *C. dubliniensis* CD48-I as follows: genomic DNA from 24 h yeast-phase *C. albicans* 132A (lane 1), cDNA from 7 h yeast-phase *C. albicans* 132A (lane 2), cDNA from 7 h hyphal-phase *C. albicans* 132A (lane 3), cDNA from 24 h yeast-phase *C. albicans* 132A (lane 4), cDNA from 24 h hyphal-phase *C. albicans* 132A (lane 5), genomic DNA from 24 h yeast-phase *C. dubliniensis* CD48-I (lane 6), cDNA from 7 h yeast-phase *C. dubliniensis* CD48-I (lane 7), cDNA from 7 h hyphal-phase *C. dubliniensis* CD48-I (lane 8), cDNA from 24 h yeast-phase *C. dubliniensis* CD48-I (lane 9) and cDNA from 24 h hyphal-phase *C. dubliniensis* CD48-I (lane 10). In the lower panel the 210 bp and 230 bp products in lane 6 and the 400 bp products in lanes 6–10, in each case, did not hybridize with the CAPLC1 probe, whereas all of the other amplimers shown in the panel did. The intensity of the amplimers obtained with the ACT primers using cDNA reverse transcribed from RNA from *C. dubliniensis* strain CD48-I cultured in the hyphal phase were consistently weaker than the corresponding amplimers obtained from yeast-phase cultures (see upper panel, lanes 7–10). (b) The PCR products shown in the lanes (both upper and lower panels) were amplified from template DNA or cDNA from *C. albicans* 132A or non-*C. albicans* *Candida* isolates from yeast phase cultures (24 h for DNA and 7 h for RNA) as follows: genomic DNA from *C. albicans* 132A (lane 1), genomic DNA from *C. tropicalis* 709A (lane 2), genomic DNA from *C. glabrata* 803A (lane 3), genomic DNA from *C. parapsilosis* 701A (lane 4), genomic DNA from *C. dubliniensis* CD48-I (lane 5), cDNA from *C. albicans* 132A (lane 6), cDNA from *C. tropicalis* 709A (lane 7), cDNA from *C. glabrata* 803A (lane 8), cDNA from *C. parapsilosis* 701A (lane 9) and cDNA from *C. dubliniensis* CD48-I (lane 10). In the lower panel the amplimer of approximately 180 bp in lane 4, the amplimers of approximately 210 bp and 230 bp in lane 5 and the 400 bp amplimers in lanes 5 and 10 did not hybridize with the CAPLC1 probe, whereas all of the other amplimers shown in the panel did. The bands <100 bp in each lane are the result of primer-dimer formation. No hybridization signal was detected in lane 8. Lanes labelled N are from PCR negative control experiments which lacked template DNA or cDNA. Lanes labelled M contain 100 bp ladder size reference markers (Gibco), the sizes of which are indicated.

C. albicans 132A was used as a PCR template; otherwise a shorter product of 156 bp would be expected. Following PCR amplification of cDNA made from 7 h and 24 h cultures of organisms grown in both the yeast and hyphal forms, a single amplimer of 210 bp was obtained in each case (Fig. 5a). No amplimers were obtained in control experiments from which reverse transcriptase was omitted. This result demonstrated that the 210 bp PCR product obtained was amplified from mRNA template reverse transcribed as cDNA and not from contaminating DNA. The 210 bp product hybridized strongly to the cloned DNA from pDB016 (encoding 72% of the CAPLC1 gene; Fig. 1) in subsequent

Southern hybridization experiments. These data demonstrated that the CAPLC1 gene is expressed in *C. albicans* 132A, that sequences encoding the unusual stretch of 18–19 amino acids within the CAPLC1 X domain are transcribed and that the gene is expressed in both the yeast and hyphal forms of the organism. This finding rules out the possibility that the DNA sequences encoding this region constitute an intron. The possibility that the unique stretch of 18–19 amino acid residues is removed from the CAPLC1 protein by intein-mediated post-translational modification was considered unlikely because it is too short to be an intein and does not contain any of the recognized motifs thought to be involved

Fig. 6. Southern blot analysis of *CAPLC1*-homologous sequences in isolates of *C. albicans* (a, b) and isolates of various non-*C. albicans* *Candida* species (c) probed with the *CAPLC1* DNA insert from pDB008 (Fig. 1). Genomic DNA was digested with *EcoRI* (a) or with *AvaI* and *SpeI* (b, c). DNA was from organisms as follows: (a) *C. albicans* 937A2 (lane 1), 928 (lane 2), 935 (lane 3), 927 (lane 4) and 132A (lane 5). (b) *C. albicans* 927 (lane 1), 928 (lane 2), 937A2 (lane 3), 935 (lane 4) and 132A (lane 5). (c) *C. albicans* 132A (lane 1), *C. tropicalis* isolates 709A and 921B (lanes 2 and 3), *C. glabrata* isolates 800A and 706B (lanes 4 and 5), *C. parapsilosis* isolates 701A and 811A (lanes 6 and 7) and *C. dubliniensis* isolates CD46 and CD48-I (lanes 8 and 9). Size reference markers consisting of a 1 kb ladder (Promega) are indicated on the left of each panel.

in splicing activity (Perler *et al.*, 1994; F. Perler, personal communication).

To determine the size of the *CAPLC1* mRNA transcript in *C. albicans* 132A, total RNA was isolated from the yeast and hyphal forms of the organism following 7 h and 20 h growth, in each case, and Northern hybridization experiments were performed using the *CAPLC* gene from pDB017 as a probe. No transcripts that hybridized with the probe were detected in each of ten separate experiments with separate preparations of RNA. In positive control experiments RNA was probed with a portion of the gene encoding elongation factor 3 (*TEF3*), and *TEF3* mRNA signals were detected from all cultures. Since *TEF3* mRNA is expressed under most conditions in yeast and hyphal forms of *C. albicans* (Swoboda *et al.*, 1994), these results suggested that *CAPLC1* mRNA is expressed at very low levels under the conditions tested, and not detectable by Northern analysis. That *CAPLC1* expression does occur under the conditions tested was demonstrated by the RT-PCR experiments described above, although it should be noted that this technique is immensely more sensitive than Northern blotting. It is possible that expression of *CAPLC1* occurs at higher levels under conditions we have not tested such as during infection *in vivo*.

Hybridization analysis of *C. albicans* with *CAPLC1*

To demonstrate that the cloned DNA contained in recombinant plasmids pDB001 and pDB008 was derived from *C. albicans* 132A, hybridization analysis was performed using the insert DNA from both plasmids

separately on *C. albicans* 132A total cellular DNA which had been digested with *EcoRI* only, with *AvaI* only, and with *AvaI* and *SpeI*. Each probe hybridized specifically to a single 20 kb *EcoRI* fragment, a 4.5 kb *AvaI* fragment, and a 3.0 kb *AvaI*-*SpeI* fragment, respectively (the data obtained with *EcoRI*-digested DNA and with *AvaI*- and *SpeI*-cleaved DNA using the pDB008 insert as a probe are shown Fig. 6a, b, lane 5). These results confirmed that the *CAPLC1* DNA cloned in pDB008 is derived from *C. albicans* 132A and is present as a single copy per genome equivalent.

EcoRI-digested genomic DNA from 26 separate *C. albicans* oral isolates was also examined by hybridization analysis using the pDB008 insert as a probe. For 14 of the isolates, the probe hybridized to a single band of approximately 20 kb with *EcoRI*-digested DNA similar to that obtained with *C. albicans* 132A, whereas 10 isolates yielded a band of approximately 15 kb in addition to a 20 kb hybridization band (Fig. 6a, lanes 1 and 3). The remaining two isolates yielded a single *EcoRI* hybridization band of 15 kb in each case (data not shown). *AvaI*- and *SpeI*-digested genomic DNA from 10 of the *C. albicans* oral isolates from AIDS patients included in the study yielded three hybridization profiles with the probe. Seven of the isolates yielded a single hybridization band of 3.0 kb similar to *C. albicans* 132A, one isolate yielded a 3.0 kb band and an additional 6.0 kb band and the remaining two isolates yielded three hybridization bands of 3.0 kb, 1.8 kb and 1.2 kb, respectively (Fig. 6b). These results indicated that in the majority of the oral isolates tested the location of *EcoRI*, *AvaI* and *SpeI* cleavage sites flanking the *CAPLC1* gene

were similarly arranged to *C. albicans* 132A. However, in some isolates polymorphisms in the sizes of CAPLC1-encoding restriction fragments affecting only one of the CAPLC1 gene homologues in each case had occurred. Similar polymorphisms have been previously reported for the *C. albicans* LIP1 gene family (Fu *et al.*, 1997).

To determine if an X domain containing the 'unique' stretch of 18–19 amino acid residues was encoded by other *C. albicans* strains, the XDOM primer set used in the RT-PCR experiments was applied to target genomic DNA from the 15 *C. albicans* oral isolates from AIDS patients. Following PCR amplification a single product of approximately 210 bp was observed in each case, similar to the result obtained with *C. albicans* 132A (data not shown). These findings demonstrated that the presence of the unique stretch of 18–19 residues was a consistent feature in *C. albicans* isolates.

C. albicans 132A chromosomes separated by PFGE were also hybridized with the 3.0 kb *AvaI*–*SpeI* CAPLC1-encoding cloned DNA from pDB008. The probe hybridized to a single chromosome band approximately 1.02 Mb in size (data not shown), which corresponded to *C. albicans* chromosome no. 7 according to the numbering system described by Magee *et al.* (1988, 1992).

Analysis of CAPLC1 homologous sequences in *Candida* species other than *C. albicans*

EcoRI-digested and *AvaI*- and *SpeI*-digested genomic DNA from eight non-*C. albicans* *Candida* species was tested for homology to CAPLC1 by hybridization analysis with the 3.0 kb CAPLC1-encoding insert DNA from pDB008. Using identical high-stringency hybridization conditions (i.e. hybridization temperature of 68 °C) to those used with the *C. albicans* isolates no or very weak hybridization signals were detected with isolates of other non-*C. albicans* *Candida* species, including *C. dubliniensis* ($n = 10$), *C. tropicalis* ($n = 5$), *C. glabrata* ($n = 11$), *C. parapsilosis* ($n = 2$), and one isolate each of *C. kefyr*, *C. krusei*, *C. guilliermondii* and *C. lusitaniae*. However, when experiments were repeated at a lower hybridization temperature (60 °C), weak hybridization signals were detected with all 10 *C. dubliniensis* isolates tested, three *C. tropicalis* isolates, one *C. glabrata* isolate and one *C. parapsilosis* isolate but with none of the *C. kefyr*, *C. krusei*, *C. guilliermondii* and *C. lusitaniae* isolates. Nine of the *C. dubliniensis* isolates tested each yielded a single *EcoRI* hybridization band of approximately 20 kb and a single *AvaI*/*SpeI* hybridization band of 3.5 kb (Fig. 6c). The remaining *C. dubliniensis* isolate, CD46, yielded a single *EcoRI* hybridization band of 15 kb and two weak *AvaI*/*SpeI* hybridization bands of approximately 3.2 and 4.8 kb, respectively (Fig. 6c). All three *C. tropicalis* isolates (709A, 803B and 915B), the *C. glabrata* isolate (803A) and the *C. parapsilosis* isolate (701A) which hybridized with the probe yielded single *EcoRI* hybridization bands, in each case, of approximately 8 kb, 3 kb and 6 kb, respectively and single *AvaI*/*SpeI* hybridization bands,

in each case, of 3.4 kb, 3.4 kb and 2.8 kb, respectively (Fig. 6c). These findings suggest that the isolates which yielded weak hybridization signals contained CAPLC1-homologous sequences.

To further explore the relationship between the *C. albicans* CAPLC1 gene and the homologous sequences detected in the non-*C. albicans* *Candida* species tested, the degenerate primers originally used to PCR amplify sequences encoding part of the X domain from genomic DNA of *C. albicans* 132A (Table 1) were applied to target genomic DNA from *C. dubliniensis* CD48-I, *C. glabrata* 803A, *C. parapsilosis* 701A and *C. tropicalis* 709A, all of which hybridized previously with the CAPLC1 probe. Following PCR amplification, several amplicons were obtained from each target DNA sample, one of which of 130 bp in each case hybridized with the CAPLC1 DNA of pDB008 (data not shown). Following purification, the 130 bp amplicon from each of the three non-*C. albicans* isolates tested, excluding *C. parapsilosis*, was cloned in pBluescript and its nucleotide sequence determined. Repeated attempts to clone the 130 bp product amplified from *C. parapsilosis* target DNA failed. For each amplicon, the sequence of 61 bases internal to primer sequences was aligned with the corresponding sequence previously amplified from *C. albicans* 132A (Fig. 7a). The sequences of the cloned amplicons from the *C. dubliniensis*, *C. glabrata* and *C. tropicalis* isolates showed extensive nucleotide homology with the corresponding sequence from *C. albicans* and with the corresponding sequences of PLC genes previously characterized from *Sacch. cerevisiae* and *Schiz. pombe* (Fig. 7a). This finding was hardly surprising as the region of DNA amplified and sequenced encoded for part of the highly conserved X domain region of PLC proteins which is essential for catalytic activity.

Genomic DNA from a representative isolate of each non-*C. albicans* *Candida* species which did not hybridize with the CAPLC1 probe was also used as a template for PCR amplification using the CAPHO primer set. Following PCR amplification, while several amplicons were obtained from each target DNA sample, none in each case hybridized with the CAPLC1 DNA of plasmid pDB008 (data not shown). This result confirmed that some but not all isolates of the same species possessed a CAPLC1-homologous sequence. The variability in the presence of detectable CAPLC1-homologous sequences in isolates of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* was very surprising. The reasons for this are unknown but it could be due to loss of common sequences following genetic rearrangements or deletion events. Failure to detect CAPLC1-homologous sequences by hybridization analysis correlated with failure to amplify CAPLC1-homologous sequences using PCR. This latter finding suggests the total absence of CAPLC1-homologous sequences or that significant differences occur in the sequence composition of PLC genes in particular isolates of non-*C. albicans* *Candida* species which failed to hybridize with the CAPLC1 probe.

(a)	
<i>C. albicans</i> 132A (CAPLC1)	1749 AACTGGTCGACAAGTTGCTGGTGATTTCATCAGTAGAAGGGTATATACGAACATTACAACGT
<i>C. dubliniensis</i> CD48-I (CDPLC1)	AACTGGTCGACAAGTTGCTGGTGATTTCATCAGTAGAAGGATATATAAGAACATTACAACGT 96.7%
<i>C. tropicalis</i> 709A	AACTGGTCGAAAAGTTGCTGGTGATTTCATCAGTAGACGGGTATATACGAACATTACAAGT 93.4%
<i>C. glabrata</i> 803A	CACTGGCAGCAAAATAGGTGGGGCACCATCAGTAGAGGGGTATATACAGGCATTACAGCAA 68.9%
<i>S. cerevisiae</i> (SCPLC)	1197 ATTGGGGAACAAATTCGAGAACTCCATCTGTGGAGGGATATATTCAAGTCCTACAACAA 59.0%
<i>S. pombe</i> (SPPLC)	1379 ACTAGGTAAGCAGTTTGGTGGAGAAAGTTCATTGAAGGTTATATCCGTTTCGCTACAGGCT 59.0%
(b)	
<i>C. albicans</i> 132A (CAPLC1)	585 GRQVAGDSSVEGYIRTLQRCRCVEIDIWNGDSNTTTTIVIGTKDDDKNEYEPVNHGRTFTFKPIS
<i>C. dubliniensis</i> CD48-I (CDPLC1)	GRQVAGDSSVEGYIRTLQRCRCVEIDIWNGDSNDTGSDV-----KNESEPIVNHGRTFTFKPIS 80.6%
	***** * *

Fig. 7. (a) Comparison of nucleotide sequences of CAPLC1-homologous amplimers obtained by PCR using template genomic DNA from *C. albicans* 132A (cloned in pDB001; Figs 1a and 2), *C. dubliniensis* CD48-I, *C. tropicalis* 709A and *C. glabrata* 803A with the degenerate CAPHO oligonucleotide primer set (Table 1) and the corresponding regions of the previously characterized PLC genes from *Sacch. cerevisiae* (SCPLC; Yoko-o *et al.*, 1993) and *Schiz. pombe* (SPPLC; Fankhauser *et al.*, 1995). Nucleotide sequences corresponding to the PCR primers are not included in the alignments. Nucleotides which differ from the *C. albicans* 132A CAPLC1 sequence are emphasized in bold. Nucleotide co-ordinates shown on the left of the figure for CAPLC1 are numbered as in Fig. 2. The nucleotide co-ordinates for the *Sacch. cerevisiae* and *Schiz. pombe* sequences are as published. Values shown on the right refer to the percentage identity of each sequence with the corresponding region from the *C. albicans* 132A CAPLC1 gene. (b) Alignment of the amino acid sequence deduced from the nucleotide sequences of cloned amplimers obtained with template genomic DNA from *C. albicans* 132A and *C. dubliniensis* CD48-I using the XDOME primer set (Table 1). These primers were specifically designed to amplify the region of DNA (210 bp) which encoded the novel stretch of 18/19 amino acids (residues underlined) present in CAPLC1 X domain. The XDOME primers amplified a CAPLC1-homologous PCR product of 180 bp with genomic DNA from *C. dubliniensis* CD48-I. The nucleotide sequence of this region from *C. dubliniensis* only encoded for 11 amino acids in the region corresponding to the *C. albicans* novel stretch of amino acids. Asterisks indicate identical residues and dots represent similar residues. Dashes indicate gaps created to obtain alignment. Residue co-ordinates shown on the left of the figure for CAPLC1 are numbered as in Fig. 2. The percentage identity of the aligned sequences is shown on the right.

Chromosome-sized DNA molecules from *C. tropicalis* 709A, *C. glabrata* 803A, *C. parapsilosis* 701A and *C. dubliniensis* CD48-I and CD46 were separated by PFGE and were hybridized with the CAPLC1 DNA of plasmid pDB008 (data not shown). The probe hybridized to single chromosome bands of approximately 1.2 Mb, 1.1 Mb and 1.0 Mb in *C. tropicalis* 709A, *C. glabrata* 803A and *C. parapsilosis* 701A, respectively. However, the probe hybridized to two chromosome bands each in the two *C. dubliniensis* isolates, a 1.0 Mb and a 2.2 Mb band in isolate CD46, and a 1.2 Mb and a 2.2 Mb band in isolate CD48-I. It is possible that the pattern observed in both *C. dubliniensis* isolates is a result of chromosome fragmentation, a phenomenon which has previously been observed in other *C. dubliniensis* karyotype profiles (Coleman *et al.*, 1997; D. Sullivan & D. C. Coleman, unpublished data).

Because all 10 of the *C. dubliniensis* isolates included in the study hybridized with CAPLC1 and because previous studies have shown that *C. dubliniensis* is the species phylogenetically most closely related to *C. albicans* (Sullivan *et al.*, 1995, 1996, 1997), experiments were performed to determine whether the *C. dubliniensis* sequences homologous to CAPLC1 encoded a similar novel stretch of amino acids as detected within the X domain of the *C. albicans* CAPLC1 protein (Fig. 4). The XDOME primer set, which was complementary to

sequences encoding this region in *C. albicans* 132A (Table 1), was used to amplify sequences from *C. dubliniensis* CD48-I and CD46 DNA. Two amplimers, of approximately 180 bp and 380 bp, respectively, were obtained in each case, only the smaller of which hybridized with the CAPLC1-encoding DNA from pDB008. Both amplimers from *C. dubliniensis* CD48-I were purified, cloned in pBluescript and their nucleotide sequences determined. The deduced amino acid sequence of the larger amplimer (359 bp) exhibited no homology with any previously characterized PLC gene in the GenBank database (analysis performed in March 1997), whereas that of the smaller amplimer (179 bp) shared 80.6% overall amino acid identity (82.1% similarity) with the corresponding *C. albicans* sequence (Fig. 7b). However, the *C. dubliniensis* sequence only encoded 11 amino acids in the region corresponding to the *C. albicans* 'novel' stretch of 19 residues, only six of which were identical and one similar to the corresponding *C. albicans* sequence (Fig. 7b).

To determine whether the CAPLC1-homologous sequences detected in non-*C. albicans* *Candida* species by hybridization and PCR analyses were expressed as mRNA, RT-PCR experiments were performed on total cellular RNA from 24 h broth cultures of one isolate of *C. dubliniensis*, and one isolate in each case of those *C. tropicalis*, *C. glabrata* and *C. parapsilosis* isolates which

exhibited *CAPLC1* homology. Amplimers of similar size to those obtained with *C. dubliniensis* CD48-I, *C. tropicalis* 709A, *C. parapsilosis* 701A genomic DNA were also obtained when cDNA made from these isolates was used as a PCR template with the XD0M primer set (Fig. 5b). These findings showed that *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* *CAPLC1*-associated DNA was present as mRNA transcripts and was therefore expressed. mRNA was also found to be present in *C. dubliniensis* isolate CD48-I following growth in both the yeast and hyphal phases at both 7 h and 24 time intervals, in each case (Fig. 5a). However, no amplimer was detected with cDNA from *C. glabrata* 803A. An amplimer of the expected size (440 bp) was obtained when the same cDNA preparation from *C. glabrata* 803A cDNA was used as a PCR template with the ACT primer set (Table 1) which served as a positive control for cDNA synthesis and RT-PCR (Fig. 5b). This suggested that the *C. glabrata* 803A *CAPLC1*-associated DNA sequence was not present as an mRNA transcript and was therefore not expressed under the conditions used.

Phospholipase C activity of *Candida* species

All of the *Candida* isolates used in hybridization experiments were tested for PLC activity using *p*-NPPC following growth in YPD for 20 h. The PLC activities in units (mg protein)⁻¹ in cell-free protein extracts of the isolates examined ranged as follows: *C. dubliniensis* (*n* = 10), 6.0–18.2 [mean 9.6 ± 3.4] × 10⁻²; *C. glabrata* (*n* = 11), 1.9–4.6 [mean 3.1 ± 0.8] × 10⁻²; *C. tropicalis* (*n* = 5), 6.3–10.9 [mean 8.2 ± 1.7] × 10⁻²; *C. parapsilosis* (*n* = 2; the individual values for each isolate are given) 4.6 and 8.6 × 10⁻², respectively. The corresponding PLC activities for the *C. albicans* oral carriage isolates tested (*n* = 3) ranged from 6.5 to 12.6 [mean 8.7 ± 2.8] × 10⁻² units (mg protein)⁻¹, whereas activities for the *C. albicans* oral isolates from AIDS patients with oral candidosis (*n* = 15) ranged from 5.4 to 15.1 [mean 11.5 ± 3.9] × 10⁻² units (mg protein)⁻¹.

Concluding remarks

We have described the genetic characterization of the first PLC gene, termed *CAPLC1*, to be cloned from the pathogenic yeast *C. albicans*. With the development of efficient gene-targeting systems in *C. albicans* it is possible to use cloned DNA sequences to generate null mutants of specific genes (Fonzi & Irwin, 1993; Gow *et al.*, 1994) and to study non-lethal mutations (Brown *et al.*, 1996). The precise role of the *CAPLC1* gene in *C. albicans* is unknown; however, based on sequence homology with previously characterized PLCδ1 genes from other yeasts and from higher eukaryotes and the presence of conserved regions within the predicted *CAPLC1* protein, it is likely that *CAPLC1* is involved in signal transduction. The presence of *CAPLC1*-related sequences in several non-*C. albicans* *Candida* species also indicated that these species contain PLC genes, which very probably perform a similar function to

CAPLC1. By disrupting the *CAPLC1* gene we hope to investigate the role of this PLCδ1 isoform in the yeast *C. albicans*. These studies and more detailed investigations of PLC genes in non-*C. albicans* *Candida* species are currently under way.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Wellcome Trust (no. 047204) and by the Dublin Dental Hospital. We thank Professor Robert Kresinger, University of Virginia, Charlottesville, USA, for his advice on EF-hand domains and Dr Francine Perler, New England Biolabs, MA, USA, for her advice on protein splicing. We would also like to gratefully acknowledge the constant encouragement and support of Professor D. Shanley (Dublin Dental Hospital).

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Received 3 July 1997; revised 22 September 1997; accepted 30 September 1997.