A *Candida albicans* cyclic nucleotide phosphodiesterase: cloning and expression in *Saccharomyces cerevisiae* and biochemical characterization of the recombinant enzyme

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We have cloned a *Candida albicans* gene, which encodes a cyclic nucleotide phosphodiesterase (PDEase), by complementation in a *Saccharomyces cerevisiae* PDEase-deficient mutant. The deduced amino acid sequence is similar to that of the low-affinity PDEase of *S. cerevisiae* (PDE1) and the cyclic nucleotide PDEase (PD) of *Dictyostelium discoideum*. Biochemical analysis of recombinant protein produced in *S. cerevisiae* indicated that the enzyme behaves as a PDE1 homologue: it hydrolyses both cAMP ($K_m = 0.49$ mM) and cGMP ($K_m = 0.25$ mM), does not require divalent cations for maximal activity and is only moderately inhibited by millimolar concentrations of standard PDEase inhibitors. Based on these data, we designate the *C. albicans* we have cloned, *PDE1*. Low-stringency genomic Southern blots showed cross-hybridization between *C. albicans PDE1* and DNA from *Candida stellatoidea*, but not with DNA from *S. cerevisiae* or several closely related *Candida* species.

Keywords: *Candida albicans*, cyclic nucleotide phosphodiesterase, morphogenesis

INTRODUCTION

The molecular basis of transition in growth between yeast (blastospore) and hyphal forms of the pathogenic fungus *Candida albicans* has been the subject of intensive research (reviewed by Odds, 1985). The ability to undergo morphogenesis is believed to confer greater pathogenicity on the organism (Sobel *et al.*, 1984) or, minimally, provide a survival advantage in the host (Shepherd, 1991). Demonstration of cyclic 3',5'-adenosine monophosphate (cAMP) involvement in morphogenesis of *Mucor* species (Cantore *et al.*, 1980; Larsen & Sypherd, 1974; Paveto *et al.*, 1975; Paznokas & Sypherd, 1975) and *Histoplasma capsulatum* (Maresca *et al.*, 1977) prompted several groups to investigate the role of cAMP in the yeast-to-hyphal transition in *C. albicans*. The results have been mixed, with reports of both increases (Niimi *et al.*, 1980; Chattaway *et*

al., 1981; Sabie & Gadd, 1992) and decreases (Egidy *et al.*, 1989) in cAMP levels just preceding or during germ tube formation; one study found no change in cAMP content during morphogenesis (Sullivan *et al.*, 1983). As in most organisms, cAMP content is regulated by the combined action of adenylate cyclase and cAMP phosphodiesterase(s) [PDEase(s)], both enzyme activities having been detected in *C. albicans* (Egidy *et al.*, 1990).

We believe that cloning genes encoding enzymes involved in regulating intracellular cAMP levels should provide tools for understanding how cAMP levels are modulated and how, if at all, they control the process of morphogenesis. Cells of *Saccharomyces cerevisiae* contain two cAMP PDEases encoded by the genes *PDE1* (Nikawa *et al.*, 1987; Suoranta & Londesborough, 1984) and *PDE2* (Londesborough & Suoranta, 1983; Sass *et al.*, 1986; Wilson & Tatchell, 1988). *PDE1* is a low-affinity (high- K_m) enzyme of 42 kDa, whereas *PDE2* is a high-affinity (low- K_m) enzyme of 61 kDa. PDEase-deficient mutants of *S. cerevisiae* are viable but sensitive to a variety of environmental stress conditions due to elevated intracellular cAMP concentrations (Colicelli *et al.*, 1989; Nikawa *et al.*, 1987; Sass *et al.*, 1986; Wilson & Tatchell, 1988). By complementation of the heat-shock sensitivity

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Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; 2-ME, 2-mercaptoethanol; PDEase, phosphodiesterase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L12045 (Gb_pl:YSAPDE1X).

Table 1. Strain list

Genotype		Source/reference
<i>S. cerevisiae</i>		
F762	<i>MATα trp1Δ1 ura3-52</i>	G. R. Fink (Whitehead Institute, Cambridge, MA)
RW134-2C	<i>MATα leu2 his3 his4 trp1 ura3 pde2::URA3</i>	Wilson & Tatchell (1988)
GL62	<i>MATα leu2 ura3-52 ade1 his3 trp1 lys2-208 pde1::LEU2 pde2::URA3 pep4::HIS3</i>	McLaughlin <i>et al.</i> (1993)
DC17	<i>MATα his1</i>	J. B. Hicks (ICOS Corp., Bothell, WA)
<i>C. albicans</i>		
B311 (ATCC 32354)		ATCC (Rockville, MD)
B792		J. A. Gorman (Bristol-Myers Squibb, Princeton, NJ)
<i>C. stellatoidea</i>		
ATCC 11006		ATCC
ATCC 36232		ATCC
<i>C. glabrata</i>		
		C. Frey (SmithKline Beecham, King of Prussia, PA)
<i>C. guilliermondii</i>		C. Frey
<i>C. keyfr</i> (ATCC 46764)		ATCC
<i>C. krusei</i>		C. Frey
<i>C. lusitaniae</i>		C. Frey
<i>C. parapsilosis</i>		C. Frey
<i>C. tropicalis</i>		C. Frey
<i>C. viswanathii</i> (ATCC 22981)		ATCC

of a PDE2-deficient *S. cerevisiae* strain, we isolated a *C. albicans* gene encoding an enzyme capable of cyclic nucleotide hydrolysis. This paper describes the molecular cloning and DNA sequence of this gene, its expression in *S. cerevisiae* and an initial characterization of the biochemical properties of the recombinant enzyme.

METHODS

Strains and media. Yeast strains used in this study are listed in Table 1. Cells of *Escherichia coli* strain DH5 α MCR (Gibco BRL) containing various plasmids were grown in Luria broth or on Luria agar plates (Sambrook *et al.*, 1989) supplemented with 100 μ g ampicillin ml⁻¹. For genomic DNA isolations, *S. cerevisiae* and *Candida* strains were grown in YEPD (Sherman *et al.*, 1986). *S. cerevisiae* strains were grown on synthetic complete medium (Hicks & Herskowitz, 1976) without leucine (SC-Leu) or tryptophan (SC-Trp) to select for plasmid maintenance.

DNA manipulations and transformations. Plasmid DNA was recovered from *E. coli* using the alkaline lysis method of Birnboim & Doly (1979). Genomic DNA from *C. albicans* and *S. cerevisiae* was prepared using the method of Sherman *et al.* (1986). Competent *E. coli* cells were prepared according to

Stoker *et al.* (1984). Plasmids were introduced into *S. cerevisiae* by either the spheroplast (Hinnen *et al.*, 1978) or lithium acetate method of transformation (Ito *et al.*, 1983).

Isolation of genomic clones by complementation in *S. cerevisiae*. PDEase-deficient mutants of *S. cerevisiae* exhibit growth-arrest phenotypes associated with elevated intracellular cAMP content, including heat-shock sensitivity (Nikawa *et al.*, 1987; Sass *et al.*, 1986; Wilson & Tatchell, 1988). Cells of the heat-shock-sensitive strain RW134-2C (*pde2::URA3*) (Table 1) were transformed with a YEp13-based *C. albicans* genomic library (Rosenbluh *et al.*, 1985). Approximately 6000 clones with an average insert size of 5–10 kb, representing 2–4 *C. albicans* genome equivalents were screened. Transformants were grown at 30 °C from regeneration agar and re-plated on SC-Leu. Colonies were replicated to SC-Leu plus 5 mM cAMP and incubated for 2 d at 30 °C; the addition of cAMP accentuates the heat-shock phenotype displayed by RW134-2C (Wilson & Tatchell, 1988). Colonies were replicated to fresh plates of the same medium which had been preheated at 55 °C for 1 h, then shifted to 55 °C for 2 h, and transferred to 30 °C; growth was monitored for 7 d.

A single colony was isolated following the heat-shock protocol and tested for co-segregation of the Leu⁺ and heat-shock-

resistance phenotype during growth on nonselective (YEPD) media. Mitotic instability was observed for both phenotypes. Plasmid DNA was recovered, reintroduced into cells of RW134-2C, and found to confer heat-shock resistance; the plasmid was designated pLH41. Subfragments of the 9 kb pLH41 insert were subcloned into YEp351 (Hill *et al.*, 1986) and retested for activity via heat shock. The insert from the smallest active subclone, a 4.5 kb *Bam*HI–*Hind*III fragment, was shuttled into pUC19 for DNA sequencing.

DNA sequencing and sequence analysis. DNA sequencing was performed by the dideoxy termination method (Sanger *et al.*, 1977), using a Sequenase kit (US Biochemical), and [³⁵S]dATP γ S (New England Nuclear). Nested deletions were constructed using the Double Stranded Nested Deletion Kit (Pharmacia) to determine the first strand sequence. The second strand was sequenced using custom oligonucleotide primers. Double-stranded template DNA was prepared according to Kraft *et al.* (1988). Acrylamide and urea gels of 8% (w/v) were run and dried on a Bio-Rad model 583 gel dryer. Dried gels were exposed to Kodak X-OMat AR film at room temperature and developed. DNA and protein sequences were analysed using GCG software (Devereux *et al.*, 1984). The PROSITE database was accessed using the MOTIFS program of GCG software (Devereux *et al.*, 1984).

Expression of *C. albicans* PDE1 in *S. cerevisiae*. *C. albicans* PDE1 was subcloned into the *S. cerevisiae* expression vector p138NB (McHale *et al.*, 1991), which is a derivative of p138 (Livi *et al.*, 1990). This expression vector contains the TRP1 selectable marker and partial 2 μ sequences for maintenance at high copy number. Expression is driven by the copper-inducible CUP1 gene promoter. An *Ava*II–*Sfi*I (filled in) fragment of pLH42 containing *C. albicans* PDE1 (Fig. 1b) was inserted into the *Nco*I–*Sa*I (filled in) sites of the multiple cloning site downstream of the CUP1 promoter and upstream of the *S. cerevisiae* CYC1 transcription terminator. Since *Ava*II cuts within the PDE1 coding region, the 5' end of the gene was repaired with annealed synthetic oligonucleotides which formed an *Nco*I site at the 5' end and *Ava*II site at the 3' end. The resulting plasmid, pLH44, was introduced into *S. cerevisiae* PDEase-deficient strain GL62 (*pde1::LEU2 pde2::URA3*) (Table 1).

Cyclic nucleotide hydrolysis assays. Cells were grown in SC–Leu liquid medium to OD₅₄₀ 1.0 and induced with 150 μ M CuSO₄ for 4 h. Cells expressing recombinant PDE1 were lysed and extracts prepared as previously described (McHale *et al.*, 1991). The resulting 100 000 g supernatant was utilized in cyclic nucleotide hydrolysis assays.

PDEase activity was assayed using a modification of the method of Davis & Daly (1979), as described previously (Torphy & Cieslinski, 1990). The reaction was initiated with either enzyme or substrate and incubated at 30 °C. Incubation time was varied depending on the amount of enzyme activity. All assays were conducted in the linear range of the reaction, where less than 20% of the initial substrate is hydrolysed. Kinetic assays were controlled so as to limit substrate hydrolysis to no greater than 10%. Kinetics were analysed with a KINPAC computer program (Cleland, 1979) using a non-linear least-squares regression analysis. K_m and V_{max} values reported are the mean of duplicate measurements on lysates of two individual clones of GL62 cells containing pLH44.

When screening compounds for PDEase inhibition, the enzyme was preincubated with the compounds for approximately 10 min at room temperature. Reactions were initiated with the substrate and the samples incubated for 30 min at 30 °C. Values reported are the mean of duplicate measurements with variations

of less than 5%. Dilution curves indicated linear hydrolysis within the range of dilution, eliminating the possible effect on enzymic activity of contaminants in cell lysates (such as residual Cu²⁺). As a control for the possible effect of cations in the cell lysis buffer, enzymic activity was measured in the presence and absence of EDTA; full enzymic activity was observed in the presence of up to 15 mM EDTA.

Southern blotting. Chromosomal DNA (5 μ g) was digested to completion with restriction enzymes and separated on 1% (w/v) agarose gels. Standard methods (Sambrook *et al.*, 1989) were used for agarose gel electrophoresis and DNA transfer to nitrocellulose membranes (Schleicher and Schuell).

Probes were prepared by random priming using the Pharmacia OligoLabelling Kit and [³²P]dCTP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; ICN). Unincorporated nucleotides were separated from labelled fragments using NICK columns (Pharmacia). Hybridizations were carried out at 42 °C in 50% (v/v) formamide (Sambrook *et al.*, 1989). Wash conditions were 2 $\times$ SSC, 0.5% (w/v) SDS at 42 °C for low stringency and 0.5 $\times$ SSC, 0.1% (w/v) SDS at 68 °C for high stringency (Sambrook *et al.*, 1989).

Fragments of the *S. cerevisiae* PDE1 and PDE2 genes which were used as probes in genomic Southern blots were obtained by PCR amplification of genomic *S. cerevisiae* DNA. Synthetic oligonucleotide primers were prepared to amplify sequences corresponding to nucleotides 213–1005 of the 1107 bp PDE1 coding region (Nikawa *et al.*, 1987) and nucleotides 439–1578 of the 1581 bp coding region of PDE2 (Sass *et al.*, 1986). Restriction sites were incorporated into the PCR primers for cloning of fragments into pUC vectors. An *Ava*II–*Sca*I fragment of pLH42 was used as a *C. albicans* PDE1 probe (Fig. 1b).

RESULTS

Isolation of a *C. albicans* cyclic nucleotide PDEase-encoding gene via complementation in *S. cerevisiae*

S. cerevisiae contains two genes encoding cAMP PDEases: PDE1, which encodes a low-affinity enzyme of 42 kDa (Londesborough & Suoranta, 1983; Sass *et al.*, 1986) and PDE2, encoding a high-affinity 61 kDa protein (Nikawa *et al.*, 1987; Suoranta & Londesborough, 1984; Wilson & Tatchell, 1988). Our initial attempt at isolating PDEase-encoding genes from *C. albicans* involved cross-hybridization with *S. cerevisiae* genes. ³²P-labelled fragments corresponding to the coding regions of the *S. cerevisiae* PDE1 and PDE2 genes failed to detect any cross-hybridizing restriction fragments in *C. albicans* genomic DNA even at low stringency (data not shown).

Our next approach involved complementation in a *S. cerevisiae* mutant strain. Cells containing null mutations in both PDE1 and PDE2, or in PDE2 alone, are viable, but exhibit specific growth-arrest phenotypes such as heat-shock sensitivity, the inability to grow under starvation conditions, or the inability to survive prolonged nitrogen starvation (Colicelli *et al.*, 1989; Nikawa *et al.*, 1987; Sass *et al.*, 1986; Wilson & Tatchell, 1988). These phenotypes are a consequence of increased intracellular cAMP levels. We utilized the heat-shock sensitivity of a PDE2-deficient *S. cerevisiae* mutant strain, RW134-2C (Wilson & Tatchell, 1988), to screen a YEp13-based *C. albicans* genomic library for a complementing clone. A single clone was isolated (after 7 d growth) capable of reversing the heat-

Fig. 1. (a) Diagram of 9 kb insert fragment from plasmid pLH41. All *Bam*HI and *Hind*III sites are included; additional *Kpn*I sites not shown in the map are present. Sites in parentheses indicate those destroyed by ligation into YEp13. The 4.5 kb *Bam*HI-*Hind*III insert, the smallest fragment tested capable of complementing the heat-shock phenotype of RW134-2C, is indicated by the lower, shaded bar. This fragment was subcloned from its original YEp351 construct, pLH42, into pUC19 to create pLH43 for DNA sequencing. (b) The 4.5 kb *Bam*HI-*Hind*III insert with restriction sites predicted from DNA sequencing. The bar below the map represents the ORF encoding *PDE1*, with the arrow indicating the direction of transcription. Locations of the *Ava*II and *Sfi*I sites used in subcloning *PDE1* for expression, the *Ava*II and *Sca*I sites used to create the *C. albicans PDE1* probe for genomic Southern analysis, and *Xho*I sites necessary for interpretation of Southern analyses are included.

shock-sensitive phenotype in RW134-2C. The activity of the transforming plasmid (designated pLH41) was confirmed by demonstrating co-segregation of heat-shock resistance and the *Leu*⁺ phenotype in cells grown in nonselective media. pLH41 also was found to confer heat-shock resistance when reintroduced into RW134-2C.

Partial restriction mapping of pLH41 indicated it contained a 9 kb insert (Fig. 1a). Fragments of the pLH41 insert digested with *Bam*HI and/or *Hind*III were subcloned into YEp351 (Hill *et al.*, 1986) to define a smaller active sub-fragment. YEp351 (5.6 kb), like YEp13 (10.7 kb), contains the *LEU2* gene as a selectable marker and 2 μ sequences for maintenance at high copy number. One plasmid, pLH42, which contains the central 4.5 kb *Bam*HI-*Hind*III fragment of the pLH41 insert (Fig. 1a) was found to be active. RW134-2C cells harbouring pLH42 grew in only 2 d following heat shock as compared to the 7 d required for growth of RW134-2C(pLH41). It is not clear why pLH42 complemented the *pde2::URA3* mutation more efficiently than pLH41. The weak complementation observed with pLH41 may be due to a lower copy number, which may be a function of its larger size (approx. 20 kb) compared to pLH42 (approx. 10 kb) (see Broach, 1983). Alternatively, the 9 kb pLH41 insert may contain sequences (absent in pLH42) that have a negative influence on copy number or plasmid maintenance. Since the *PDE1* open reading frame (ORF) is located in the centre of the 4.5 kb insert in pLH42 (see

below), it is unlikely that any specific *cis*-acting sequences required for transcriptional repression were eliminated during subcloning.

DNA sequence of the complementing clone

The entire complementing 4.5 kb *Bam*HI-*Hind*III fragment was sequenced and found to contain a long ORF (Fig. 1b) predicting a 426-amino-acid protein (Fig. 2) with identity to known PDEases (Fig. 2), especially with the low-affinity cAMP PDEase of *S. cerevisiae* (Nikawa *et al.*, 1987) and the cyclic nucleotide PDEase, PD, of the slime mould *Dictyostelium discoideum* (Lacombe *et al.*, 1986). A multiple alignment of these three sequences is shown in Fig. 3. Based on the extensive similarities between the *C. albicans* and *S. cerevisiae* proteins, we designated the gene that we have cloned *PDE1*. Since, in theory, expression of any enzyme capable of hydrolysing cAMP would have been detected in our complementation assay, it is not surprising that the low-affinity cAMP PDEase from *C. albicans* was found to complement the phenotype associated with elevated cAMP content caused by a lack of the high-affinity enzyme. In fact, cDNAs encoding several different types of mammalian cAMP PDEases have been cloned via complementation in yeast (Colicelli *et al.*, 1989; Michaeli *et al.*, 1993).

Although the three sequences shown in Fig. 3 display several regions with a high degree of conservation, the overall percent identity at both the nucleotide and amino acid level was rather low (Table in Fig. 3). Values of 44% identity at the nucleotide level between the *S. cerevisiae* and *C. albicans PDE1* sequences may account for the failure to isolate the gene by cross-hybridization.

The 5' untranslated region (UTR) of *PDE1* contains some elements frequently observed in eukaryotic promoters (Fig. 2), including two putative TATA elements at -34 and -66 and CCAATCT (with one mismatch) at -72 (Nussinov, 1990). The sequences adjacent to the putative AUG codon compare favourably with a consensus sequence (AAAAAAUGUCU) derived from the analysis of many *S. cerevisiae* genes (Hamilton *et al.*, 1987). Several putative polyadenylation signals, all with a single mismatch from the consensus AATAAA (Proudfoot & Brownlee, 1976), are present within the 3' UTR.

Structural features of the deduced *PDE1* protein

Analysis of the PROSITE database for consensus sequences in *C. albicans PDE1* associated with a biological function revealed a sequence with similarity to a motif called a cAMP PDEase class-II signature which is present in both *S. cerevisiae PDE1* and *D. discoideum PD*, but not in any of the other PDEases sequenced to date including several mammalian enzymes, the *Drosophila dnc* gene product, and *S. cerevisiae PDE2* (Fig. 4a) (Beavo & Reifsnyder, 1990; Charbonneau *et al.*, 1986). This signature sequence was not derived from any residues shown to be of importance for catalysis but was chosen due to the fact that three conserved histidine residues are found in this region. Differences between the consensus sequence and *C. albicans PDE1* amino acid sequence are indicated in Fig.

-300 GTGATTAACGTGTACACGACGACGACGACGACGATCGCGCCTTAATC
 -249 CTAAGACAAAAAAGAAACCGGTACAACTCTTGAATTCCTCCATT
 -198 CTGTGGTTCCTCACTAAAAAAGATTAAAAAGAACACACCGGAAATCGA
 -147 AGCAATAAATTAATAATTAATTTTAACTCACTCCGGAACAAACGAA
 -96 AAAAAAAGAACTCTCTCTCAACCAAACTATATCGTTATTATTCAAGA
 -45 AAAAAAATTTATATCTCTCTTCAACATAATAGCAAACTAAATGTCA
 1 M S
 7 TTTGAAATCACAATCTTGGGTCGTCAGGAGGTCCATTAGAGGGAAGCACT
 3 F E I T I L G S S G G P L E G S T
 58 TGTTCAAATCTTCTAAAACCCGCAACATCTCTTATCAGCATCATCAAT
 20 C S I L L K P A N I S Y H D I I N
 109 GACAATCTACCTGATCAAGTATTATGTATCGATGCTGGATCAGGTATGGG
 37 D N L P D Q V L C I D A G S G M G
 160 AAATAACCGAGATTATTCATCAGGAACACGACTAAGACTTCATATTGT
 54 K L T E I I H Q E T T T K T S Y C
 211 AATTTCTTCAATATTCTGATGTGAAACCGGTCTTATTATTATCAT
 71 N F L Q Y Y P D C E T V S Y Y Y H
 262 CCTAATGTAACCAATACCCATTTTCAACCTTCAACGAGTAGACCG
 88 P N V T I T T P F S N F O P G R P
 313 ATTCTACATACCCAGAAATTTTCAATAACCTACAAAATTACCTTATTC
 105 I L H T Q N L Q N Y L I S
 364 CATTCCTATTGGATGATVTTGTTCATGATAAATTCTCGTGGATT
 122 H S H L D H V C S V V I N S A G F
 415 AATAAAATATGCTGAATAAAATTTATATGGATCTCATTACAGCAATAAC
 139 N K N M L M N K I L Y G S H Y T I N
 466 GCAATGCAACCAATTTATTCAGGGGAAAGTTTGCCCAATATGCTAGT
 156 A M Q Q H L F N G K V W P N M P S
 517 TTCAAAATCGTCAATTTAACTATTGAAACCAATAGAAAGTGAAGAATA
 173 F K I V N L N Y L E S N R S E R I
 568 GGAATATATACAGTGAATTTGATTGAGTCAAGAGAGTTTCAACAAA
 190 G I Y T V K M F D L S H G E F N K
 619 TTGACTGAAGACAGGAGACGACCAACCATAGCAATAGTAACAGTAAC
 207 L T E D K E D A Q H H S N S N S
 670 AGTAATAACATCTGGGGAAACGTTATGACCGCAGGCGATCATCAATTACT
 224 S N N I W G K R Y D R R R S I T
 721 ACCATACCTCAAAACACTAGTGGTTTGAATATAAGAAATTCGAGGCTTGT
 241 T I P Q N T S G L I I K N S E A L
 772 AACCATCATTATTTATCATGCTTTTGTATCACTAGAAAGTGGCTTGC
 258 N H H Y L S S A F L I T L E V P C
 823 ACAATTAAGGAACCCCTAGCATATTAGTATTGGAGATTTTGAAAGT
 275 T T K E P P P S I L V F G D F E S
 874 GATCTAACAGTAATTTGTCGCAACTTATTTATGGAAGAGTATAGCA
 292 D L T S K L S R N L F I W K S I A
 925 CTGTAATATTGAGAAACCAATTTGAAAGCAATCGTGTGGAGTCTTCAAT
 309 L L I L R N Q L K A I V L E C S N
 976 TGTAAGAGATAGTGCACCAAGTAATATGATGATGACACCAAAATG
 326 C K E I A A N E L Y G H L T P K L
 1027 TTAATTTATGAATTGAAACCAATTTGGAACATGAATGTAACAATTAGACCC
 343 L I Y E L K Q L E H E C K Q L D T
 1078 GCCACCACTAGTGAACCACTTATAGGGTTGAATGCTGTAATAT
 360 A T T S T E Q P L L G L N V I V N
 1129 CATGTCAAGGAACCCCTAGTGCACCAATCAGGAAGTCAATACATGAT
 377 H V K E P I A D P N Q E S Q L H D
 1180 CCTCGAAAAAGGATTTAGTGAATTAATTAATTAATGAGATAGAAAA
 394 P R K R I L A E L N L N E I E K
 1231 TTAGGATGAATATTCAATTTGCCCTTAGTGGCACCTCAATTATAGTATAG
 411 L G C N I S I A L S G T S I I V End
 1282 AGTATTGGAATAACGCCACTACAGAAATACGATGAATCCAAATATATTT
 1333 ATTAATATCTCTTCTACTTTTCAATTTTCAATTTTGCAGATTTATTT
 1384 TTAATATACAATTTTATACAACCTGTTAATTTCTTTTGTATTATTTA
 1435 TTCTCTCTGCTCATATTGTTTCTTATACAACCTCTTCTTGTCTATCT
 1486 ATTGGATAACATAAT

Fig. 2. Nucleotide sequence of *C. albicans* PDE1, with predicted amino acid translation. Putative regulatory elements frequently found in eukaryotic promoters are noted, including TATA underlined at positions -34 and -66 and CCAATCT underlined twice (with one mismatch) at -72. The cAMP PDEase class-II signature motif is underlined twice in the amino acid sequence.

4(a) by arrows. Based on these data, we propose that the class-II signature sequence be modified to accommodate the amino acid sequence predicted by *C. albicans* PDE1.

The database also indicated the presence of a leucine zipper motif in *S. cerevisiae* PDE1, a less well-matched leucine zipper in *C. albicans* PDE1 and no evidence of this motif in the *D. discoideum* sequence (Fig. 4b) (Landschulz *et al.*, 1989). *S. cerevisiae* PDE1 has been demonstrated to exist as a dimer (Londesborough & Suoranta, 1983), but the leucine zipper was not determined to be the means by which dimerization occurs. Although this conserved motif was detected in *S. cerevisiae* PDE1, the region

C.a. 1 MSFTITLIS SGPPLGSR SIAKPAIS YHIDINLF 40
 S.c. 1 M VVFTITLIS NGGPEYGD CFIKAPART DPILLI 36
 D.d. 1 MALNKKLISL LILFIFINI VNSHQEDD CDDO DEDIGISAER 42

C.a. 41 DQVLCHDAGS LKCHMETH QETTKTSYC NFD YYP CRYVSYR 88
 S.c. 37 AVDGA LKMLHMLV QGRNENEGD ELAPSTYEDH RPLPFTIDS 82
 D.d. 43 SERRSKNSN DGSFTYNG NDYVPEPNWN HYSCTATKO CRDASYTIT 90

C.a. 89 NITITLIFS NFOGRP DLHONLFW 114
 S.c. 83 KLANCKLSK SFLSL KR QPHLSANT MKKYLTFQS 120
 D.d. 91 LSTGGLIDE GNLSFLTK KGNLFIALD AGFVWGVR TITFKYIN 138

C.a. 115 LKN LLD SHSLHDVCS VVINSAGF 138
 S.c. 121 LTD LLD SHSLHDVCS LVNPSIDE 146
 D.d. 139 LFNITPSWA VLPEQTSWF LKHHVMSYH SHSLHDVCS LIIVSPEDL 188

C.a. 139 NKN MLNKL YSHYTINAM 157
 S.c. 147 QEN SKRTI MGLPHTDV 165
 D.d. 189 AKNIDVQPP INNGIMGLIR KLGFKPTDFT SSSILKRTI MGLPHTIN 238

C.a. 158 LKLFNGKVV FNFSP K IVNNYLESN RSEKICVTV KMFDLSHGE 204
 S.c. 166 KRVFNLLIW FDI DARRR KLKIKCLNPK EVCKCTIFFW DVIPFK 210
 D.d. 239 STNLFNQQW FNLSPGRV YFSIAGSIEY PFTELPPNA TTMSLVAN 288

C.a. 205 NKLTEDADA CRRSSNNS NNDWGRFDR RRSITTIPQ NTSGLIKN 254
 S.c. 211 VHBIGVGC APYSTYFIP S 231
 D.d. 289 PFSVKVDFE LCBELISTE 308

C.a. 255 EALNHLYSS AFLITLVEPC LKPPPSHI VFCDFESDI TSGLRNLFI 303
 S.c. 232 LKPPPSHI VFCDFESDI TSGLRNLFI 260
 D.d. 309 FLF LKPPPSHI VFCDFESDI TSGLRNLFI 339

C.a. 304 MKSLALLR NOLKAIVLEC SNCKEIAANE LYGLHPLN LKLEKLEHE 353
 S.c. 261 MSYVAPNIP VHLKGLIVEC SCPLSSKPEQ LYGLHPLN LKLEKLEHE 310
 D.d. 340 IYANQKQIM DKLATITET SFNNPDSAS MFLHPRER MKLMDLLVQ 389

C.a. 354 CKLDATTS TROPFLSNV TVNVKRELA DPNCQLHD FRRITAEIN 403
 S.c. 311 YN SKGLSGLNV TVNVKRELA K RD FRTITAEIN 345
 D.d. 390 SIQ FSP PMTNLKHVIL TVEHNPQA S DPNSWT TCRVYQQL 432

C.a. 404 KNDIEKLC NTSIALST SHIV 426
 S.c. 346 FLAEENLGD KRISIALSGH TLFL 369
 D.d. 433 EANNNG VRTIFNOSD PUCI 452

	C.a.	S.c.	D.d.
C.a.	-	44 %	42 %
S.c.	33 %	-	41 %
D.d.	25 %	24 %	-

Fig. 3. Multiple alignment of the amino acid sequences of *C. albicans* PDE1 (C.a.), *S. cerevisiae* PDE1 (S.c.), and *D. discoideum* PD (D.d.) as generated by the PILEUP program of GCG software (Devereux *et al.*, 1984). Identical and conservatively replaced amino acids are boxed and shaded. Conservative replacements were judged using the following rules: A = G; D = E; Q = N; S = T; K = R; I = L = V. The table shows percentage nucleotide and amino acid identities between *C. albicans* PDE1 (C.a.), *S. cerevisiae* PDE1 (S.c.), and *D. discoideum* PD (D.d.). Values on the upper diagonal represent nucleotide identities from the optimal alignments generated using the GAP program of GCG software (Devereux *et al.*, 1984). Values on the lower diagonal are amino acid identities tabulated by hand using the optimal alignment generated by the GAP program (Devereux *et al.*, 1984).

containing the putative leucine zipper was not predicted to assume α -helical conformation by predictive algorithms (Devereux *et al.*, 1984). The α -helical conformation was

Fig. 4. (a) Consensus sequence for the PDEase class-II signature sequence from the PROSITE database and corresponding occurrences of this motif in *S. cerevisiae* PDE1 (S.c.), *D. discoideum* PD (D.d.), and *C. albicans* PDE1 (C.a.). Positions where any amino acid is acceptable are indicated by 'x' and positions where conservative replacements are allowed are noted by a list of alternatives. Arrows indicate the positions where the *C. albicans* sequence fails to conform to the consensus sequence. (b) Schematic diagram of predicted *S. cerevisiae* PDE1 (S.c.) and *C. albicans* PDE1 (C.a.) sequences. Regions corresponding to putative leucine zipper motifs are boxed and sequences from these regions expanded below. Residues required for leucine zipper formation are highlighted in boldface type.

Table 2. cAMP and cGMP hydrolysis

Strain	Plasmid	PDEase activity (units)*	
		cAMP hydrolysis	cGMP hydrolysis
GL62	p138NB	0	0
DC17	None	0.078	0.015
GL62	pLH44	2.1	2.5
RW134-2C	YEp13	0.023	0.013
RW134-2C	pLH41	0.024	0.014
RW134-2C	pLH42	1.8	1.7

* 1 unit = 1 nmol cNMP hydrolysed min⁻¹ (mg protein)⁻¹ at 30 °C. Assays were done in duplicate with differences of less than 5% between replicates.

predicted for the region containing the less well-matched leucine zipper pattern in *C. albicans* PDE1, but the heptad leucine repeat may not be well enough conserved to be functional (Fig. 4b).

D. discoideum PD is an extracellular enzyme secreted by means of a classical signal peptide (Lacombe *et al.*, 1986). There is no evidence for an N-terminal signal peptide in

Table 3. Effect of divalent cations on recombinant PDEase activity

Cation	Concn (mM)	Specific activity (units)*	% of control
Control	—	374	100
Ca ²⁺	1	358	96
	10	366	98
Cu ²⁺	1	45	12
	10	10	3
Mg ²⁺	1	378	101
	10	384	103
Mn ²⁺	1	364	97
	10	368	98
Zn ²⁺	1	94	25
	10	23	6

* 1 unit = 1 nmol cAMP hydrolysed min⁻¹ (mg protein)⁻¹ at 30 °C. Reactions contained 0.5 mM cAMP as substrate. Assays were done in duplicate with differences of less than 5% between replicates.

either *C. albicans* PDE1 or *S. cerevisiae* PDE1, consistent with their intracellular localization (Fujimoto *et al.*, 1974; Gunasekaran *et al.*, 1976).

Expression of *C. albicans* PDE1 in *S. cerevisiae*

Demonstration that the *C. albicans* PDE1 gene product hydrolyses cAMP was achieved following expression in *S. cerevisiae*. The gene was subcloned into the *S. cerevisiae* expression vector p138NB which contains the copper-inducible *CUP1* promoter. The resulting plasmid (pLH44) was introduced into PDEase-deficient *S. cerevisiae* strain GL62 (Table 1) and expression was induced by growing cells in the presence of CuSO₄. Cell lysates were assayed for cyclic nucleotide hydrolysis (Table 2). Whereas cells of GL62 harbouring p138NB alone showed no detectable cAMP or cGMP hydrolysis activity, cells with pLH44 showed a 27-fold increase in cAMP hydrolysis activity compared to the untransformed wild-type strain DC17. Furthermore, p138NB, unlike pLH44, was incapable of complementing the heat-shock-sensitive phenotype of GL62 (data not shown).

Lysates of RW134-2C cells carrying the original pLH41 complementing plasmid contained nearly background (i.e. endogenous PDE1) enzymic activity, whereas pLH42, which contains the smallest active subclone, conferred expression levels comparable to pLH44. This may account for the observed delay in growth following the heat shock assay with pLH41 (7 d) versus pLH42 (2 d). These data suggest that only a slight reduction in intracellular cAMP levels is required to reverse the heat-shock phenotype in strain RW134-2C. Thus, there is a significant difference between the amount of enzyme required *in vivo* to break down enough cAMP to allow cells to survive heat shock, and the amount of enzyme required to detect cAMP hydrolysis in our *in vitro* assay.

Table 4. Effect of different compounds on recombinant PDEase activity

Compound	Concn (mM)	Specific activity (units)*	% of control
Control	–	414	100
EDTA	1	404	98
	10	476	115
	15	426	103
EGTA	1	403	97
	10	383	93
2-ME	1	325	79
	10	130	31
DTT	1	67	16
	10	29	7
Aminophylline	1	473	114
	10	141	34
IBMX	1	352	85
	10	273	66
Imidazole	1	417	101
	10	345	86
Caffeine	1	517	125
	10	211	51

* 1 unit = 1 nmol cAMP hydrolysed min⁻¹ (mg protein)⁻¹ at 30 °C. Reactions contained 0.5 mM cAMP as substrate. Assays were done in duplicate with differences of less than 5% between replicates.

Biochemical characterization of recombinant *C. albicans* PDE1 expressed in *S. cerevisiae*

K_m and V_{max} values were determined by assay of lysates of *S. cerevisiae* PDEase-negative cells in which *C. albicans* PDE1 was expressed. Using cAMP as substrate, K_m and V_{max} for the recombinant enzyme were (mean $\pm$ SE) 0.49 ($\pm$ 0.048) mM and 1172 ($\pm$ 118) nmol min⁻¹ (mg protein)⁻¹, respectively. A lower K_m of 0.25 ($\pm$ 0.022) mM was calculated for cGMP as substrate with a V_{max} of 44 ($\pm$ 2.7) nmol min⁻¹ (mg protein)⁻¹. The double reciprocal plots for all studies were linear with $r^2 = 0.99$. Within a single experiment, the standard errors were always $\leq 10\%$ of the calculated kinetic constants.

Divalent cation requirements of this enzyme were also investigated (Table 3). Addition of cations at 1 and 10 mM concentrations indicated that Ca²⁺, Mg²⁺ and Mn²⁺ had essentially no effect on *C. albicans* PDE1 activity, while Cu²⁺ and Zn²⁺ were strongly inhibitory. Addition of EDTA and EGTA (Table 4) also did not significantly alter *C. albicans* PDE1 activity. Moderate and strong inhibition were noted in the presence of 2-mercaptoethanol (2-ME) and dithiothreitol (DTT), respectively.

The PDEase inhibitors aminophylline and 3-isobutyl-1-methylxanthine (IBMX) had moderate inhibitory effects on the activity of *C. albicans* PDE1 (Table 4). Imidazole and caffeine were also moderate inhibitors of enzyme

activity. *C. albicans* PDE1 activity was not stimulated by addition of Ca²⁺/calmodulin (data not shown). Heating of the cell lysate at 100 °C for 1 min completely inactivated *C. albicans* PDE1 activity.

Occurrence of PDE1 in related species

The occurrence of *C. albicans* and *S. cerevisiae* PDE1 homologues in known pathogenic *Candida* species and organisms demonstrated by rRNA sequencing to be most closely related to *C. albicans* (Barns *et al.*, 1991) was investigated using Southern blots of genomic DNA. *S. cerevisiae* PDE1 hybridized to its corresponding genomic *Xho*I fragment, but failed to detect genomic sequences from *C. albicans* and *C. stellatoidea* (Fig. 5a). An identical blot probed with an internal *Ava*II–*Sca*I fragment of *C. albicans* PDE1 showed a lack of hybridization with *S. cerevisiae* DNA, but detected a single fragment in DNA from *C. albicans* and *C. stellatoidea* (Fig. 5b). All other *Candida* species tested (*C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitanae*, *C. parapsilosis*, *C. tropicalis* and *C. viswanathii*) showed no cross-hybridization using either gene as a probe. These data suggest that either these organisms do not have PDE1-like genes, or, as is the case for the PDE1 genes of *C. albicans* and *S. cerevisiae*, nucleotide conservation between the genes of the various species is below the point at which it can be detected by low-stringency hybridization.

DISCUSSION

We have isolated a *C. albicans* gene (PDE1), encoding a cyclic nucleotide PDEase, by complementation in a *S. cerevisiae* PDEase-deficient mutant. Sequence comparisons indicated that *C. albicans* PDE1 is a homologue of *S. cerevisiae* PDE1 and shares identity with the extracellular cAMP PDEase of *D. discoideum*. The conclusion that the PDE1 proteins from *C. albicans* and *S. cerevisiae* are homologues is further substantiated by the close similarity of their biochemical characteristics. K_m values calculated for the *C. albicans* enzyme compare favourably with the K_m of 0.25 mM for cAMP and 0.16 mM for cGMP measured for *S. cerevisiae* PDE1 (Fujimoto *et al.*, 1974); Londesborough (1982) calculated a slightly lower K_m of 0.13 mM for the *S. cerevisiae* enzyme in the presence of cAMP. The effects of divalent cations on *C. albicans* and *S. cerevisiae* PDE1 activity are essentially identical, with Ca²⁺, Mg²⁺ and Mn²⁺ being neither required nor inhibitory, strong inhibition observed in the presence of Cu²⁺ and Zn²⁺, and no effect in the presence of 10 mM EDTA (Fujimoto *et al.*, 1974; Londesborough, 1982). Londesborough & Suoranta (1983) concluded that a tightly bound Zn²⁺ ion accounted for the insensitivity of *S. cerevisiae* PDE1 to EDTA; this has yet to be investigated for the *C. albicans* enzyme. Similar to results for *C. albicans* PDE1, inhibition of the *S. cerevisiae* enzyme was observed in the presence of millimolar concentrations of 2-ME, with stronger inhibition at similar concentrations of DTT (Fujimoto *et al.*, 1974) and moderate inhibition in the presence of 10 mM theophylline (45%) and 10 mM caffeine (20%).

Fig. 5. Duplicate Southern blots of *Xho*I-digested genomic DNA from *S. cerevisiae*, *C. albicans*, and *C. stellatoidea*. (a) Probed with PCR-amplified fragment of *S. cerevisiae* PDE1. (b) Probed with *Avall*-*Sca*I internal fragment from *C. albicans* PDE1. Lanes: 1, *C. albicans* B792; 2, *C. albicans* B311 (ATCC 32354); 3, *C. stellatoidea* ATCC 11006; 4, *C. stellatoidea* ATCC 36232; 5, *S. cerevisiae* F762.

Although the amino acid sequence of *D. discoideum* PD is similar to that of the PDE1 genes, its K_m for cAMP is 5 μ M, and when complexed with the phosphodiesterase inhibitor, PDI, the K_m increases to 2 mM (Kessin *et al.*, 1979). The regulation of *D. discoideum* PD expression has been the subject of intensive study due to its involvement in development of the organism (Podgorski *et al.*, 1989); however, biochemical data for this enzyme are less abundant. In one study, *D. discoideum* PD was shown to have a higher affinity for cAMP than for cGMP (Van Ments-Cohen & Haastert, 1989). Its extracellular localization and low K_m for cAMP suggest that although *D. discoideum* PD, *C. albicans* PDE1 and *S. cerevisiae* PDE1 share a high degree of amino acid sequence similarity, biochemically, the *S. cerevisiae* and *C. albicans* enzymes are much more similar to each other than to *D. discoideum* PD.

Comparison of our biochemical data with those previously determined for PDEases in *C. albicans* is complicated by the fact that previous measurements were obtained from whole-cell lysates containing more than one PDEase activity. The presence of more than one *C. albicans* PDEase activity was noted by Gunasekaran *et al.* (1976), who calculated K_m values for cAMP of 4.2 and 250 μ M by assaying ammonium sulfate fractions of cell lysates. Our K_m value of 490 μ M confirms our identification of the cloned gene as encoding the high- K_m *C. albicans* isozyme.

Egidy *et al.* (1990) reported partial purification and characterization of one *C. albicans* PDEase isozyme with a K_m for cAMP of between 0.5 and 0.8 μ M and a high specificity for cAMP. PDEase activity was enhanced by addition of 5 mM Mg^{2+} or Mn^{2+} and inhibited 60% by 5 mM theophylline or 4 mM IBMX. The biochemical properties of this enzyme compare favourably with those

obtained from whole-cell lysates (Gunasekaran *et al.*, 1976) and are similar to those exhibited by the low- K_m PDEase of *S. cerevisiae* encoded by PDE2 (Londesborough, 1975, 1982; Sass *et al.*, 1986; Suoranta & Londesborough, 1984; Wilson & Tatchell, 1988). Based on these observations and data presented in this communication, biochemical characteristics of PDEases in *C. albicans* appear to closely resemble those reported in *S. cerevisiae*, where PDE1 and PDE2 account for total cellular PDEase activity (Nikawa *et al.*, 1987).

Since a minimum of two PDEase activities have been identified in *C. albicans*, our complementation assay, which screened several genome equivalents, should have revealed the gene encoding the high-affinity isozyme. In theory, all genes encoding enzymes which serve to decrease intracellular cAMP concentrations should have been detected. With its higher affinity for cAMP, the *C. albicans* homologue of *S. cerevisiae* PDE2 should have performed better in this capacity than *C. albicans* PDE1. It is possible that *C. albicans* PDE2 was not represented in the YEp13 genomic library utilized, that the promoter is nonfunctional in *S. cerevisiae*, or that when present on a high-copy-number vector, the high-affinity PDEase is lethal to *S. cerevisiae* cells. Attempts to isolate the second PDEase-encoding gene from *C. albicans* are in progress.

The relationship between intracellular cAMP levels and *C. albicans* morphogenesis is controversial (Chattaway *et al.*, 1981; Egidy *et al.*, 1990; Niimi *et al.*, 1980; Sabie & Gadd, 1992; Sullivan *et al.*, 1983). The biochemical data presented herein, as well as those of Egidy *et al.* (1990), demonstrate that, minimally, millimolar concentrations of methylxanthines (theophylline, aminophylline and IBMX) and caffeine are required for moderate inhibition of both *C. albicans* PDEase isozymes. These data support

the observations of a moderate rise in intracellular cAMP observed by Chattaway *et al.* (1981) in the presence of 5 mM theophylline, but not the increased intracellular cAMP noted in the presence of micromolar theophylline and caffeine concentrations by Sabie & Gadd (1992). Data derived from the use of millimolar concentrations of methylxanthines must also be interpreted cautiously since high concentrations of these compounds may affect enzymes other than PDEases (Pall, 1981).

While changes in intracellular cAMP content may occur concomitant with cellular differentiation, it remains unclear whether such changes represent a cause or effect of the process of development. Direct evidence to support or refute a role for cAMP in *C. albicans* morphogenesis can be obtained by construction of a PDEase-deficient strain and evaluation of its ability to form hyphae. Further information could be gained by following expression of *PDE1* during the yeast-to-hyphal transition. However, initial attempts to quantify expression on Northern blots have proven difficult due to low abundance of *PDE1*-specific message in *C. albicans* cells (L. L. Hoyer, unpublished observation). Since reporter gene constructs are becoming available for *C. albicans* (Leuker *et al.*, 1992), these could be utilized to study the activity of the *PDE1* promoter throughout morphogenesis, and also the effect on expression of various media and inducers of the yeast-hyphal transition.

Finally, it is worth noting that, unlike the high-affinity PDEase isozyme which was described as highly specific for cAMP hydrolysis (Egidy *et al.*, 1990), our enzyme hydrolyses cGMP with a higher affinity than for cAMP. This raises the possibility that *PDE1* may play a larger role in regulation of intracellular cGMP levels than it does for cAMP. The isolation of *C. albicans PDE1* is an important step toward gaining an understanding of the role of cyclic nucleotides in morphogenesis.

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