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


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 \( K_m = 0.49 \text{mM} \) and cGMP \( K_m = 0.25 \text{mM} \), 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 to the organism (Sobel, reviewed by Odds, 1985). The ability to undergo morphogenesis is believed to confer greater pathogenicity to the organism (Sobel, 1985; Platt & Platt, 1974; Paveto 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 & Londenborough, 1984) and *PDE2* (Londenborough & 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 (Celic et al., 1989; Nikawa et al., 1987; Sass et al., 1986; Wilson & Tatchell, 1988). By complementation of the heat-shock sensitivity
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 DH5αMCR (Gibco BRL) containing various plasmids were grown in Luria broth or on Luria agar plates (Sambrook *et al.*, 1989) supplemented with 100 μg 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 YEpl3-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 (YPEPD)
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 (Hilt et al., 1986) and retested for
activity via heat shock. The insert from the smallest active
subclone, a 4.5 kb BamHI–HindIII fragment, was shuttled into
pUC19 for DNA sequencing.

**DNA sequencing and sequence analysis.** DNA sequencing
was performed by the dyeoxy termination method (Sanger et
al., 1977), using a Sequenase kit (US Biochemical), and
[35S]ATPαs (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
GGC 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 2μ sequences for maintenance at
high copy number. Expression is driven by the copper-inducible
CUP1 gene promoter. An AvaII–Sfl (filled in) fragment of
pLH42 containing C. albicans PDE1 (Fig. 1b) was inserted into
the NorI–Sel (filled in) sites of the multiple cloning site
downstream of the CUP1 promoter and upstream of the S.
cerevisiae CYC1 transcription terminator. Since AvaII cuts
within the PDE1 coding region, the 5’ end of the gene was
repaired with annealed synthetic oligonucleotides which formed
an NorI site at the 5’ end and AvaII site at the 3’ end. The
resulting plasmid, pLH44, was introduced into S. cerevisiae
PDEase-deficient strain GL62 (p138NB: LEU2 pde2::URA3)
(Table 1).

**Cyclic nucleotide hydrolysis assays.** Cells were grown in
SC–Leu liquid medium to OD660 10 and induced with 150 μM
CuSO4 for 4 h. Cells expressing recombinant PDE1 were lysed
and extracts prepared as previously described (McHale et al.,
1991). The resulting 100000 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 &
Gieslinski, 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 enzyme 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. Km and Vmax 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
enzymatic activity of contaminants in cell lysates (such as residual
Cu²⁺). As a control for the possible effect of cations in the cell
lysate buffer, enzymatic activity was measured in the presence
and absence of EDTA; full enzymatic activity was observed in the
presence of up to 15 mM EDTA.

**Southern blotting.** Chromosomal DNA (5 μg) 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 (300 Ci mmol⁻¹, 11 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 X SSC, 0.5% (w/v) SDS at 42°C for low stringency and 0.5 X
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 blotting were obtained by
PCR amplification of genomic S. cerevisiae DNA. Synthetic
oligonucleotide primers were prepared to amplify sequences
conforming 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 AvaII–Sel 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-

C. albicans cyclic nucleotide phosphodiesterase

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**Fig. 1.** (a) Diagram of 9 kb insert fragment from plasmid pLH41. All BamHI and HindIII sites are included; additional KpnI sites not shown in the map are present. Sites in parentheses indicate those destroyed by ligation into YEp13. The 4.5 kb BamHI–HindIII 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 BamHI–HindIII insert with restriction sites predicted from DNA sequencing. The bar below the map represents the ORF not shown in the map are present. Sites in parentheses indicate encoding PDE1, with the arrow indicating the direction of transcription. Locations of the Avall and SfiI sites used in subcloning pLH41 for expression, the Avall and SfiI sites used to create the C. albicans PDE1 probe for genomic Southern analysis, and XhoI sites necessary for interpretation of Southern analyses are included.

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 BamHI and/or HindIII 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 2p sequences for maintenance at high copy number. One plasmid, pLH42, which contains the central 4.5 kb BamHI–HindIII 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 BamHI–HindIII 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 5' 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 & Reifsnnyder, 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.
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., 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 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
predicted for the region containing the less well-matched leucine zipper pattern in \textit{C. albicans} PDE1, but the heptad leucine repeat pattern may not be well enough conserved to be functional (Fig. 4b).

\textit{D. discoideum} PD is an extracellular enzyme secreted by means of a classical signal peptide (Lacombe \textit{et al.}, 1986). There is no evidence for an N-terminal signal peptide in either \textit{C. albicans} PDE1 or \textit{S. cerevisiae} PDE1, consistent with their intracellular localization (Fujimoto \textit{et al.}, 1974; Gunasekaran \textit{et al.}, 1976).

**Expression of \textit{C. albicans} PDE1 in \textit{S. cerevisiae}**

Demonstration that the \textit{C. albicans} PDE1 gene product hydrolyses cAMP was achieved following expression in \textit{S. cerevisiae}. The gene was subcloned into the \textit{S. cerevisiae} expression vector p138NB which contains the copper-inducible \textit{CUP1} promoter. The resulting plasmid (pLH44) was introduced into PDEase-deficient \textit{S. cerevisiae} strain GL62 (Table 1) and expression was induced by growing cells in the presence of CuSO\textsubscript{4}. 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 \textit{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 \textit{in vitro} assay.

**Table 3. Effect of divalent cations on recombinant PDEase activity**

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<th>Cation</th>
<th>Concen (mM)</th>
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*1 unit = 1 nmol cAMP hydrolysed min\textsuperscript{-1} (mg protein)\textsuperscript{-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.
activity. *C. albicans* PDE1 activity was not stimulated by addition of Ca\(^{2+}\)/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 *XbaI* fragment, but failed to detect genomic sequences from *C. albicans* and *C. stellatoidea* (Fig. 5a). An identical blot probed with an internal *AraI*-StuI 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. lusitaniae*, *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. *Km* values calculated for the *C. albicans* enzyme compare favourably with the *Km* 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 *Km* of 0.15 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 Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) being neither required nor inhibitory, strong inhibition observed in the presence of Cu\(^{2+}\) and Zn\(^{2+}\), and no effect in the presence of 10 mM EDTA (Fujimoto et al., 1974; Londesborough, 1982). Londesborough & Suoranta (1983) concluded that a tightly bound Zn\(^{2+}\) 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%).

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

*Km* 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, *Km* and *V*\(_{max}\) for the recombinant enzyme were (mean ± SE) 0.49 (±0.048) mM and 1172 (±118) nmol min\(^{-1}\) (mg protein\(^{-1}\))\(^{-1}\), respectively. A lower *Km* of 0.25 (±0.022) mM was calculated for cGMP as substrate with a *V*\(_{max}\) of 44 (±27) nmol min\(^{-1}\) (mg protein\(^{-1}\))\(^{-1}\). The double reciprocal plots for all studies were linear with *r*\(^2\) = 0.99. Within a single experiment, the standard errors were always ≤ 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 Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) had essentially no effect on *C. albicans* PDE1 activity, while Cu\(^{2+}\) and Zn\(^{2+}\) 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

### Table 4. Effect of different compounds on recombinant PDEase activity

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</tbody>
</table>

*1 unit = 1 nmol cAMP hydrolysed min\(^{-1}\) (mg protein\(^{-1}\))\(^{-1}\) at 30 °C. Reactions contained 0.5 mM cAMP as substrate. Assays were done in duplicate with differences of less than 5% between replicates.
Although the amino acid sequence of similar to that of the PDE1 genes, its 5 pM, and when complexed with the phosphodiesterase been the subject of intensive study due to its involvement in development of the organism (Podgorski 1979). The regulation of biochemically, the S. Robertson-Cohen C. albicans, S. cerevisiae & Haastert, 1989). Its extracellular localization and low Km for cAMP suggest that although D. discoideum PD, 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 Km values for cAMP of 42 and 250 μM by assaying ammonium sulfate fractions of cell lysates. Our Km value of 490 μM confirms our identification of the cloned gene as encoding the high-Km isozyme. Egidy et al. (1990) reported partial purification and characterization of one C. albicans PDEase isozyme with a Km for cAMP of between 0.5 and 0.8 μM and a high specificity for cAMP. PDEase activity was enhanced by addition of 5 mM Mg2+ or Mn2+ 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-Km 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 YEpl3 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

**Fig. 5.** Duplicate Southern blots of Xhol-digested genomic DNA from S. cerevisiae, C. albicans, and C. stellatoidea. (a) Probed with PCR-amplified fragment of S. cerevisiae PDE1. (b) Probed with Avai-Scal 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 762.
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. I. Hoyer, unpublished observation). Since reporter gene constructs are becoming available for C. albicans (Leukaer 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.

ACKNOWLEDGEMENTS

The authors would like to thank Carrie Frey and Kelly Tatchell for supplying strains, Jessica Gorman for providing the B792 genomic library, Carl Hoyer for his expertise in drawing figures, and Catherine Pachuk for ideas and lively discussions.

REFERENCES


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Received 18 August 1993; revised 30 November 1993; accepted 13 January 1994.