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 ($K_m = 0.49$ 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 to the organism (Sobel, 1974; Paveto et al., 1980; Chattaway, 1980; Lansdown & Londeborough, 1984) 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.
Table 1. Strain list

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
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<td>S. cerevisiae</td>
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<td></td>
</tr>
<tr>
<td>F762</td>
<td>MATα trplΔ1 ura3-52</td>
<td>G. R. Fink (Whitehead Institute, Cambridge, MA)</td>
</tr>
<tr>
<td>RW134-2C</td>
<td>MATα leu2 his4 trpl1 ura3 pde2::URA3</td>
<td>Wilson &amp; Tatchell (1988)</td>
</tr>
<tr>
<td>GL62</td>
<td>MATα leu2 ura3-52 ade1 his3 trpl1 lys-208 pde1::LEU2 pde2::URA3 pep4::HIS3</td>
<td>McLaughlin et al. (1993)</td>
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<tr>
<td>DC17</td>
<td>MATα his1</td>
<td>J. B. Hicks (ICOS Corp., Bothell, WA)</td>
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<tr>
<td>C. albicans</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>ATCC (Rockville, MD)</td>
</tr>
<tr>
<td>B792</td>
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<td>J. A. Gorman (Bristol-Myers Squibb, Princeton, NJ)</td>
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<td>ATCC</td>
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<td>ATCC</td>
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<tr>
<td>ATCC 36232</td>
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<td>ATCC</td>
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<tr>
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<td>C. Frey (SmithKline Beecham, King of Prussia, PA)</td>
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<td>C. viswanathi</td>
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<td>ATCC</td>
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<td>(ATCC 22981)</td>
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</table>

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 DH5aMCR (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 (YEPED)
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 restested 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]dATP×s (New England Nuclear). Nested deletions were
constructed using the Double Stranded Nested Deletion Kit
(Promega) 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
GCC software (Devereux et al., 1984). The PROSITE database
was accessed using the motifs program of GCC 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 TRPI
selectable marker and partial 2μ sequences for maintenance at
high copy number. Expression is driven by the copper-inducible
CUP1 gene promoter. An AscI–SfiI (filled in) fragment of
pLH42 containing C. albicans PDE1 (Fig. 1b) was inserted into
the NotI–SalI (filled in) sites of the multiple cloning site
downstream of the CUP1 promoter and upstream of the S.
cerevisiae CYC1 transcription terminator. Since AscI cuts
within the PDE1 coding region, the 5′ end of the gene was
repaired with annealed synthetic oligonucleotides which formed
an NcoI site at the 5′ end and an AvaII 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 O.D. 600 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 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. 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
enzymic activity of contaminants in cell lysates (such as residual
Cu3+). As a control for the possible effect of cations in the cell
lysate 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 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
Oligo Labelling Kit and [35P]dCTP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; ICN). Unincorporated nucleotides were
separated from labelled fragments using NICK columns
(Promega). 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 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 AvaII–SalI 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-
L. L. HOYER and OTHERS

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The transforming plasmid (designated pLH41) was found to be active. RW134-2C cells harbouring the 4.5 kb BamHI–HindIII fragment of the pLH41 insert (Fig. 1a) were found to confer heat-shock resistance when reintroduced into RW134-2C. It is not clear why pLH42 complemented the phenotype 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 (AAAAAACUGUCU) 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.

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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 with restriction sites predicted from DNA sequencing. The arrow indicating the direction of transcription.

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**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).

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which dimerization occurs. Although this conserved motif in the leucine zipper was not determined to be the means by which dimerization occurs.

The database also indicated the presence of a leucine zipper motif in C. albicans PDE1, with predicted amino acid translation. Putative regulatory elements frequently found in eukaryotic promoters are noted, including TATA box underlined twice in the amino acid sequence. Putative regulatory elements frequently found in eukaryotic promoters are noted, including TATA box underlined twice in the amino acid sequence.

The a-helical conformation was assumed by predictive algorithms 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 assumed by predictive algorithms.
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

<table>
<thead>
<tr>
<th>Strain</th>
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<th>PDEase activity (units)*</th>
<th>cAMP hydrolysis</th>
<th>cGMP hydrolysis</th>
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<td>GL62</td>
<td>p138NB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DC17</td>
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<td>0.015</td>
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</tr>
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*1 unit = 1 nmol cAMP hydrolysed min⁻¹ (mg protein)⁻¹ at 30 °C. Assays were done in duplicate with differences of less than 5% between replicates.

Table 3. Effect of divalent cations on recombinant PDEase activity

<table>
<thead>
<tr>
<th>Cation</th>
<th>Conc. (mM)</th>
<th>Specific activity (units)*</th>
<th>% of control</th>
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<tbody>
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<td>Zn²⁺</td>
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<tr>
<td></td>
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</table>

*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**

<table>
<thead>
<tr>
<th>Compound</th>
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<th>% of control</th>
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</tr>
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</tr>
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<td>DTT</td>
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<td>7</td>
</tr>
<tr>
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<td>125</td>
</tr>
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*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**

*Kₘ and Vₘₙₐₓ values were determined by assay of lysates of S. cerevisiae PDEase-negative cells in which C. albicans PDEI was expressed. Using cAMP as substrate, Kₘ and Vₘₙₐₓ for the recombinant enzyme were (mean ± SE) 0.49 (±0.048) mM and 1172 (±118) nmol min⁻¹ (mg protein)⁻¹, respectively. A lower Kₘ of 0.25 (±0.022) mM was calculated for cGMP as substrate with a Vₘₙₐₓ of 44 (±2.7) nmol min⁻¹ (mg protein)⁻¹. The double reciprocal plots for all studies were linear with r² = 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 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 XhoI fragment, but failed to detect genomic sequences from C. albicans and C. stellatoidea (Fig. 5a). An identical blot probed with an internal AvaII-ScaI 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 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%).
Although the amino acid sequence of similar to that of the PDE1 genes, its $K_m$ for cAMP is 5 $\mu$M, and when complexed with the phosphodiesterase inhibitor, PDI, the $K_m$ increases to 2 mM (Kessin et al., 1979). The regulation of \textit{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, \textit{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 \textit{D. discoideum} PD, \textit{C. albicans} PDE1 and \textit{S. cerevisiae} PDE1 share a high degree of amino acid sequence similarity, biochemically, the \textit{S. cerevisiae} and \textit{C. albicans} enzymes are much more similar to each other than to \textit{D. discoideum} PD.

Comparison of our biochemical data with those previously determined for PDEases in \textit{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 \textit{C. albicans} PDEase activity was noted by Gunasekaran et al. (1979), who calculated $K_m$ values for cAMP of 42 and 250 $\mu$M by assaying ammonium sulfate fractions of cell lysates. Our $K_m$ value of 490 $\mu$M confirms our identification of the cloned gene as encoding the high-$K_m$ \textit{C. albicans} isozyme.

Egidy et al. (1990) reported partial purification and characterization of one \textit{C. albicans} PDEase isozyme with a $K_m$ for cAMP of between 0.5 and 0.8 $\mu$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 \textit{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 \textit{C. albicans} appear to closely resemble those reported in \textit{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 \textit{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 \textit{C. albicans} homologue of \textit{S. cerevisiae} PDE2 should have performed better in this capacity than \textit{C. albicans} PDE1. It is possible that \textit{C. albicans} PDE2 was not represented in the YEp13 genomic library utilized, that the promoter is nonfunctional in \textit{S. cerevisiae}, or that when present on a high-copy-number vector, the high-affinity PDEase is lethal to \textit{S. cerevisiae} cells. Attempts to isolate the second PDEase-encoding gene from \textit{C. albicans} are in progress.

The relationship between intracellular cAMP levels and \textit{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 \textit{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. I. 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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