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


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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, 1980; Larsen, 1987). The results have been mixed, with reports of both increases (Niimi et al., 1980; 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-Km) enzyme of 42 kDa, whereas PDE2 is a high-affinity (low-Km) 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

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 (Km = 0.49 mM) and cGMP (Km = 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
of a PDE2-deficient \textit{S. cerevisiae} strain, we isolated a \textit{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 \textit{S. cerevisiae} and an initial characterization of the biochemical properties of the recombinant enzyme.

\section*{METHODS}

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

\subsection*{DNA manipulations and transformations.} Plasmid DNA was recovered from \textit{E. coli} using the alkaline lysis method of Birnboim \& Doly (1979). Genomic DNA from \textit{C. albicans} and \textit{S. cerevisiae} was prepared using the method of Sherman \textit{et al.} (1986). Competent \textit{E. coli} cells were prepared according to Stoker \textit{et al.} (1984). Plasmids were introduced into \textit{S. cerevisiae} by either the spheroplast (Hinnen \textit{et al.}, 1978) or lithium acetate method of transformation (Ito \textit{et al.}, 1983).

\section*{Isolation of genomic clones by complementation in \textit{S. cerevisiae}.} PDEase-deficient mutants of \textit{S. cerevisiae} exhibit growth-arrest phenotypes associated with elevated intracellular cAMP content, including heat-shock sensitivity (Nikawa \textit{et al.}, 1987; Sass \textit{et al.}, 1986; Wilson \& Tatchell, 1988). Cells of the heat-shock-sensitive strain RW134-2C (pde2::\textsc{ura}\textsc{3}) (Table 1) were transformed with a YEpl3-based \textit{C. albicans} genomic library (Rosenbluh \textit{et al.}, 1985). Approximately 6000 clones with an average insert size of 5-10 kb, representing 2-4 \textit{C. albicans} genome equivalents were screened. Transformants were grown at 30 °C from regeneration agar and re-plated on SC\texthyphen Leu. Colonies were replicated to SC\texthyphen 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\textsuperscript{+} and heat-shock-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Genotype & Source/reference & \\
\hline
\textit{S. cerevisiae} F762 & MAT\textalpha trpl1 ura3-52 & G. R. Fink (Whitehead Institute, Cambridge, MA) \\
RW134-2C & MAT\alpha leu2 his3 his4 trpl ura3 pde2::\textsc{ura}\textsc{3} & Wilson \& Tatchell (1988) \\
GL62 & MAT\alpha leu2 ura3-52 ade1 his3 trpl lys2-208 pde2::\textsc{leu}\textsc{2} pep4::\textsc{his}\textsc{3} & McLaughlin \textit{et al.} (1993) \\
DC17 & MAT\alpha his1 & J. B. Hicks (ICOS Corp., Bothell, WA) \\
\textit{C. albicans} B311 (ATCC 32354) & & ATCC (Rockville, MD) \\
B792 & & J. A. Gorman (Bristol-Myers Squibb, Princeton, NJ) \\
\textit{C. stellatoidea} ATCC 11006 & & ATCC \\
ATCC 36232 & & ATCC \\
\textit{C. glabrata} & & C. Frey (SmithKline Beecham, King of Prussia, PA) \\
\textit{C. glabrata} (ATCC 46764) & & ATCC \\
\textit{C. krusei} & & C. Frey (SmithKline Beecham, King of Prussia, PA) \\
\textit{C. lusitaniae} & & C. Frey (SmithKline Beecham, King of Prussia, PA) \\
\textit{C. parapsilosis} & & C. Frey (SmithKline Beecham, King of Prussia, PA) \\
\textit{C. tropicalis} & & C. Frey (SmithKline Beecham, King of Prussia, PA) \\
\textit{C. viswanathii} (ATCC 22981) & & ATCC \\
\hline
\end{tabular}
\caption{Strain list}
\end{table}
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 restested for activity via heat shock. The insert from the smallest active subclone, a 4.5 kb BamHI–HindII 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]dATPαs (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 Kuch 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 3μ sequences for maintenance at high copy number. Expression is driven by the copper-inducible CUP1 gene promoter. An Aval–Sfl (filled in) fragment of pLH42 containing C. albicans PDE1 (Fig. 1b) was inserted into the Nol–SalI (filled in) sites of the multiple cloning site downstream of the CUP1 promoter and upstream of the S. cerevisiae CYC1 transcription terminator. Since AvalI cuts within the PDE1 coding region, the 5’ end of the gene was repaired with annealed synthetic oligonucleotides which formed an Nol site at the 5’ end and AvalI site at the 3’ end. The resulting plasmid, pLH44, was introduced into S. cerevisiae PDE1-deficient strain GL62 (pdel::LEU2 pde2::URA3) (Table 1).

**Cyclic nucleotide hydrolysis assays.** Cells were grown in SC – Leu liquid medium to OD_{600} 1.0 and induced with 10 μM CuSO₄ for 4 h. Cells expressing recombinant PDE1 were lyzed and extracts prepared as previously described (McHale et al., 1991). The resulting 10000 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. 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 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 [32P]dCTP (3000 Ci 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 × SSC, 0.5% (w/v) SDS at 42 °C for low stringency and 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 Aval–Sfl 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-
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 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 Dictostelium 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 AAATAA (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 indicated. Additional Kpnl 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 encoding PDE1, with the arrow indicating the direction of transcription. Locations of the Avall and Sfcl sites used in subcloning PDE1 for expression, the Avall and Scal sites used to create the C. albicans PDE1 probe for genomic Southern analysis, and Xhol sites necessary for interpretation of Southern analyses are included.
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 in C. albicans PDE1, a less well-matched leucine zipper in S. cerevisiae 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 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

![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 and CCAATCT underlined twice (with one mismatch) at -72. The CAMP PDEase class-II signature motif is underlined twice in the amino acid sequence.](image-url)

![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 = 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 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).](image-url)
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 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 levels required for cAMP 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 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>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td>-</td>
<td>374</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺</td>
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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.
C. albicans cyclic nucleotide phosphodiesterase

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

Table 4. Effect of different compounds on recombinant PDEase activity

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<th>Compound</th>
<th>Conc (%)</th>
<th>Specific activity (units)*</th>
<th>% of control</th>
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<td></td>
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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.

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 recombinent enzyme were (mean ± se) 0.49 (±0.048) mM and 1172 (±118) nmol min⁻¹ (mg protein)⁻¹, respectively. A lower K_m 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. 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 XbaI–SalI 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. 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 0.15 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 D. discoideum PD is 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 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 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- $K_m$ 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 $\mu$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 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...
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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REFERENCES


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