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A *Candida albicans* homolog of a human cyclophilin gene encodes a peptidyl-prolyl *cis-trans* isomerase

(Recombinant DNA; heterologous gene expression; rotamase; cyclosporin A; phage λ library)

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SUMMARY

A *Candida albicans* cDNA and its genomic counterpart were isolated from λ phage libraries using a human T-cell cyclophilin (Cyp) cDNA as a hybridization probe. The clones contain a 486-bp open reading frame predicting a 162-amino acid, approx. 18 kDa protein which is similar in size to, and which shares 68 and 81% homology with, human T-cell Cyp and cytosolic *Saccharomyces cerevisiae* Cyp, respectively. Northern blots show the presence of a single mRNA species of about 800 bp. However, genomic Southern blots suggest the presence of at least one other Cyp-related gene in *C. albicans*. The cDNA was engineered for expression in *Escherichia coli*, and the resulting recombinant protein, like mammalian Cyps, exhibited a peptidyl-prolyl *cis-trans* isomerase (PPIase) activity which was sensitive to inhibition by cyclosporin A in vitro. These results indicate that the gene which we have cloned encodes a *C. albicans* Cyp. We designate this gene *CYP1* (cyclophilin). Interestingly, the predicted *C. albicans* protein contains only two cysteine residues which do not align with any of the four cysteines conserved among mammalian Cyps. This suggests that the PPIase catalytic mechanism may not involve an enzyme-bound hemithioorthoamide, as previously reported for porcine Cyp.

INTRODUCTION

Cyclophilin (Cyp) is an 18-kDa protein expressed abundantly in the cytosol of most if not all eukaryotic tissues (Koletsky et al., 1986). Cyp was so named because it binds

cyclosporin A (CsA), a cyclic undecapeptide produced by *Tolypocladium inflatum*. CsA was originally identified as an antifungal compound but has since proven to be a powerful immunosuppressive agent (Stahelin, 1986). Recently, Cyp was reported to be a peptidyl-prolyl *cis-trans* isomerase (PPIase) which promotes the folding of oligopeptides and proteins at prolyl-peptide bonds in vitro (Fischer et al., 1989b; Takahashi et al., 1989). The proline isomerization catalyzed by Cyp is strongly inhibited by the binding of CsA (Fischer et al., 1989b; Takahashi et al., 1989). While the endogenous role of Cyp is not known, it may be required for the maturation of receptors (Schneuwly et al., 1989) or regulatory factors (Emmel et al., 1989). As it has been proposed that the immunosuppressive action of CsA is due to the inhibition of PPIase activity, it may also be involved in the regulation of intracellular signaling events leading to T-cell activation (Emmel et al., 1989).

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); *C.*, *Candida*; CsA, cyclosporin A; Cyp, cyclophilin; *CYP1*, gene encoding *C. albicans* Cyp; *D.*, *Drosophila*; DTT, dithiothreitol; *E.*, *Escherichia*; kb, kilobase(s) or 1000 bp; *N.*, *Neurospora*; nt, nucleotide(s); ORF, open reading frame; PA, polyacrylamide; PAGE, PA-gel electrophoresis; pfu, plaque-forming units; PCR, polymerase chain reaction; PPIase, peptidyl-prolyl *cis-trans* isomerase; *S.*, *Saccharomyces*; SDS, sodium dodecyl sulfate; YEPD, yeast extract peptone dextrose.

A cDNA encoding Cyp was originally isolated from human T-cells (Haendler et al., 1987), having a deduced aa sequence of 165 residues in length. cDNAs have since been cloned from five other highly divergent eukaryotic organisms, including rat (Danielson et al., 1988), Chinese hamster (Bergsma and Sylvester, 1990), *Drosophila melanogaster* (Shieh et al., 1989), *Neurospora crassa* (Tropschug et al., 1988), and *Saccharomyces cerevisiae* (Haendler et al., 1989; Koser et al., 1990). These proteins exhibit between 45 and 96% aa sequence homology with human Cyp. Southern blot analysis of human and rat genomic DNA has revealed up to 20 copies of Cyp-homologous sequences, which indicates the existence of multiple related genes or pseudogenes (Danielson et al., 1988; Haendler et al., 1987). Northern blot analysis of rat RNA has shown that Cyp mRNA is present at high levels in nearly all tissues. However, in *Drosophila*, the expression of the *ninaA* gene, which encodes a Cyp isozyme, is tissue specific (Schneuwly et al., 1989; Shieh et al., 1989). Also, in contrast to other CyPs, the *Drosophila* protein has a potential transmembrane domain. Although the function of this protein is unknown, it has been hypothesized to be involved in the posttranscriptional control of rhodopsin levels in the cell membrane.

N. crassa has been found to produce both cytosolic and mitochondrial forms of Cyp from a single gene having four introns (Tropschug, 1990; Tropschug et al., 1988). In *S. cerevisiae*, a cytosolic form of Cyp has been isolated and shown to possess PPIase activity, albeit at 10% the enzymatic level of the human form (Haendler et al., 1989). However, the binding of CsA to *S. cerevisiae* Cyp is identical to the binding to human Cyp (Haendler et al., 1989).

Furthermore, Cyp has been shown to be the cellular target that mediates CsA cytotoxicity in both *S. cerevisiae* and *N. crassa* (Tropschug et al., 1989). We have recently cloned a second, actively transcribed *S. cerevisiae* Cyp-encoding gene (Koser et al., 1990). The protein predicted from this sequence exhibits 52% and 56% identity with the human and cytosolic *S. cerevisiae* CyPs, respectively. In addition, it contains a hydrophobic N-terminal extension, which may function as a signal sequence for subcellular compartmentalization.

Cyp-related genes have also been isolated from the prokaryotes *Escherichia coli* (Kawamukai et al., 1989) and *Salmonella typhimurium* (Tran et al., 1990). Interestingly, the *E. coli* Cyp gene product is found in the periplasm and is not inhibited by CsA (Liu and Walsh, 1990).

We initiated the present work to gather basic information on a *Candida albicans* Cyp gene and its protein product as a preliminary step toward the understanding of the physiologic role of CyPs in pathogenic yeast. The exogenously produced *C. albicans* cyclophilin constitutes a useful tool for further enzymatic and biochemical studies of PPIase catalysis and for the design of PPIase inhibitors.

RESULTS AND DISCUSSION

(a) Cloning and characterization of Cyp-related genomic and cDNA sequences

Four overlapping clones were isolated from a *C. albicans* cDNA library by virtue of homology to a human T-cell Cyp probe. Using one cDNA fragment as a probe, two homologous sequences were subsequently isolated from a *C. albicans* genomic library. Both genomic clones appeared to be identical based on restriction mapping (data not shown). A composite of the genomic and cDNA sequences, along with the deduced aa sequence, are shown in Fig. 1. Although each of the four cDNAs begins at a different position in the 5'-untranslated region, all were found to contain a 486-bp ORF, encoding a 162-aa protein of 17575 Da (Fig. 1). Furthermore, all four cDNAs were found to be colinear with the genomic sequence (no introns). Each cDNA exhibits a 3'-untranslated region of about 110 bp. In addition, one cDNA clone was found to possess a poly(A) tract (not shown), beginning immediately after nt + 599. The 3'-untranslated region of each cDNA contains a potential polyadenylation signal sequence AATAA at a position 26 bp upstream from the polyadenylation site (Fig. 1). The genomic sequence also contains a probable TATA element (TATATA) beginning at position -126. We designate the *C. albicans* Cyp gene that we have cloned *CYP1*.

(b) Evidence for multiple Cyp-related sequences in *Candida albicans*

Northern blot analysis shows that the *CYP1* cDNA recognizes an abundant approx. 800-nt mRNA present in both vegetative yeast-phase cells (blastospores) and fully differentiated mycelia (Fig. 2). The size of the mRNA compares favorably with the size of the cDNA. Despite the presence of what appears to be a single *CYP1* mRNA species, genomic Southern blots indicate the presence of additional sequences homologous to the cDNA (Fig. 3). At present, it is not known whether these additional sequences represent functional genes or pseudogenes. However, it is unlikely that all of the additional bands reflect polymorphic differences between two homologues of the same gene in this diploid organism. The existence of an additional *C. albicans* Cyp-related gene is quite possible, given that two Cyp genes have been isolated from *S. cerevisiae* (Haendler et al., 1989; Koser et al., 1990). The single band of positive hybridization in our RNA blot suggests that if additional *C. albicans* Cyp genes exist and are actively transcribed, the transcript size cannot be significantly different from that of *CYP1*.

(c) Analysis of the deduced *CYP1* aa sequence

Alignment of the predicted *C. albicans* *CYP1* aa sequence with the sequences of CyPs from other organisms shows

-538
 AAAAGCTTTTTCATGGCAGTCTTAGTAAAGAACTATAGCAATTAAGATCTGATGTAATATA -475
 ATCCCATTAATCTAGATTACGTTTACATTTAAATTCACACCCCAATTCATCAATAGGGTCAGACACACAC -396
 ACACATGAGGTAATGTTGAAAAAGCAATCTGTTCAGACACTACTTTTTCATACAATGTTCCAGCAGCTTCTATT -317
 TGGCAAGATTTACACGACCTGTGTGCGTGGCTTCTTGTGCTGTCTCATTCCACACCCCTTAAAGTTGAA -238
 AACGACGATGTTGTTGTCGACAAAATCTCTAAACAGCTAATTAACAATGGAAGACACACATATGAAAACCTCAACA -159
 AACGAAGAACGAAGAAAAAAATTTCAATATATTTTGGACATTTAAATCTTCTCTCTTCTCTCTTCTTCTT -80
 CTTCCTCTTCTTCTTCTTCTTCTAAGTTCATTTGTTACTTGAATACTCAATTACTACCAAGAACACCCCAATAAAA -1
 Met Ser Thr Val Tyr Phe Asp Val Ser Ala Asp Gly Gln Lys Leu Gly Lys Ile Thr Phe 20
 ATG TCT ACC GTT TAC TTT GAT GTC TCC GCT GAT GGT CAA AAA TTA GGA AAG ATC ACC TTC 60
 Cys Leu Tyr Asp Asp Val Val Pro Lys Thr Ala Glu Asn Phe Arg Ala Leu Cys Thr Gly 40
 AAA TTG TAC GAT GAT GTC GTT CCA AAG ACT GCT GAA AAT TTC AGA GCT TTA TGT ACT GGT 120
 Glu Lys Gly Phe Gly Tyr Lys Gly Ser Ile Phe His Arg Val Ile Pro Gln Phe Met Leu 60
 GAA AAA GGT TTT GGT TAC AAA GGT TCT ATT TTC CAC AGA GTC ATC CCA CAA TTC ATG CTT 180
 Gln Gly Gly Asp Phe Thr Asn Phe Asn Gly Thr Gly Gly Lys Ser Ile Tyr Gly Thr Lys 80
 CAA GGT GGT GAC TTC ACC AAC TTT AAC GGT ACT GGT GGT AAA AGT ATT TAC GGT ACC AAA 240
 Phe Ala Asp Glu Asn Phe Val Lys Arg His Asp Arg Pro Gly Leu Leu Ser Met Ala Asn 100
 TTT GCT GAT GAA AAC TTT GTC AAG AGA CAT GAC AGA CCA GGT TTG TTG TCT ATG GCC AAT 300
 Ala Gly Pro Asn Thr Asn Gly Ser Gln Phe Phe Ile Thr Thr Val Pro Cys Pro Trp Leu 120
 GCT GGT CCA AAC ACC AAT GGT TCC CAA TTC TTC ATT ACC ACC GTT CCA TGC CCA TGG TTG 360
 Asp Gly Lys His Val Val Phe Gly Glu Val Thr Asp Gly Leu Asp Ile Val Lys Lys Ile 140
 GAT GGT AAA CAC GTT GTT TTC GGT GAA GTC ACT GAT GGT TTA GAC ATT GTT AAA AAG ATT 420
 Glu Ser Phe Gly Ser Gly Ser Gly Ala Thr Ser Lys Lys Ile Val Ile Glu Glu Ser Gly 160
 GAA TCT TTT GGT TCT GGC AGC GGT GCT ACT TCT AAG AAG ATT GTC ATT GAA GAA TCT GGT 480
 Gln Leu *** 162
 CAA TTG TAA GTGAAGCACGAAGAAAAGTAATTAATAGTTACTATATTTTTTTTAGGTCTTTTATAGCTTTATG 556
 TCAAGATGAAAAATAACAACCAAAAGGGAATTAATCTCAGTTAAGAATTTGTATTGTAAGATGAAAAAGTTGGGC 635
 AACGACCTGAAGGTGGTGGATAATGGAGGAAGCAATCTGTGATGTATGGAGTGTGCTAATGTC 704

1 2

9.49-
7.46-
4.40-

2.37-

1.35-

0.24-

Fig. 1.

Fig. 2.

Fig. 1. Nucleotide sequence of *C. albicans* CYP1 and deduced aa sequence. The *C. albicans* cDNA library was constructed and screened using the following procedure. Poly(A)⁺ RNA was isolated from culture of *C. albicans* blastospores following standard methods (Aviv and Leder, 1972; Carlson and Botstein, 1982). Double-stranded cDNA was synthesized from 2 µg of poly(A)⁺ RNA using a cDNA synthesis kit (Boehringer-Mannheim). The method was modified to include a filling-in reaction as the last step. After the addition of *Eco*RI linkers, the double-stranded cDNA was cloned into the *Eco*RI site of Lambda-Zap (Stratagene, La Jolla, CA). The titer of the library was 1.5×10^6 pfu/ml. Phage plaques were transferred to nitrocellulose and were screened by hybridization to a 524 bp PCR-generated fragment corresponding to human T-cell Cyp cDNA (Haendler et al., 1987). The cDNA probe was labeled using [α -³²P]dCTP (ICN, Irvine, CA) and a random primer labeling kit (Bethesda Research Laboratories, Gaithersburg, MD). DNA from positive plaques was excised to yield recombinant Bluescript plasmids in *E. coli* XL-1 Blue cells (Stratagene). Four cDNA clones were isolated and sequenced. Cyp genomic clones were isolated from a *C. albicans* genomic library constructed in a YEpl3-based plasmid vector (Rosenbluh et al., 1985). Ampicillin-resistant *E. coli* colonies containing sequences homologous to the ³²P-labeled *C. albicans* Cyp cDNA were identified by colony hybridization (Maniatis et al., 1982). Two plasmids with identical inserts (based on partial restriction mapping) were identified. The nt sequence above is a composite of genomic and cDNA sequences (GenBank accession No. M37101). The stop codon is denoted by ***. Each of four cDNA clones exhibits a different 5' terminus (denoted by arrows). A potential TATA element (TATATA, shown in box) is located at a position 126 nt upstream from the translation initiation site. The putative polyadenylation signal (AATAA) is underlined, and the polyadenylation site (+ 599) is denoted with an open arrowhead. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method (Sanger et al., 1977) with modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH). For genomic and cDNA clones, both strands were sequenced at least twice, using synthetic oligodeoxynucleotide primers.

Fig. 2. Northern blot analysis of Cyp gene transcripts in blastospores and mycelia. Cells of *C. albicans* strain B311-A were grown to mid-log phase (approx. 1×10^8 cells/ml) in YEPD broth (Hicks and Herskowitz, 1976) at 30°C; under these conditions cells existed primarily as blastospores (vegetative budding cells). To induce mycelial differentiation, cells were first grown in YEPD to mid-log phase, harvested, washed once with sterile water, and resuspended at 5×10^6 cells/ml in RPMI-1640 plus glutamine (GIBCO, Grand Island, NY). Cultures were incubated at 37°C without shaking in 5% CO₂ for 16 h; at this time nearly 100% of the cells had differentiated into mycelia. Aliquots of poly(A)⁺ RNA from blastospores and mycelia were size fractionated by electrophoresis through 1.5% ME agarose (SeaKem, FMC, Rockland, ME) in the presence of 2.2 M formaldehyde (Lehrach et al., 1977). Each lane contained 2 µg of poly(A)⁺ RNA from either mycelia (lane 1) or blastospores (lane 2). Samples were transferred to nitrocellulose and hybridized to a 800-bp ³²P-labeled *C. albicans* Cyp cDNA fragment containing the complete coding region. Hybridizations were carried out at 65°C in 6 × SCP (0.6 M NaCl/0.18 M Na₂HPO₄/6 mM EDTA, pH 6.2) containing 1% N-lauryl sarcosine/10% dextran sulfate 45 µg herring sperm DNA per ml. Filters were washed at 65°C in 2 × SCP/0.5% SDS, and autoradiographed. Sizes were estimated relative to internal RNA size markers (Bethesda Research Laboratories).

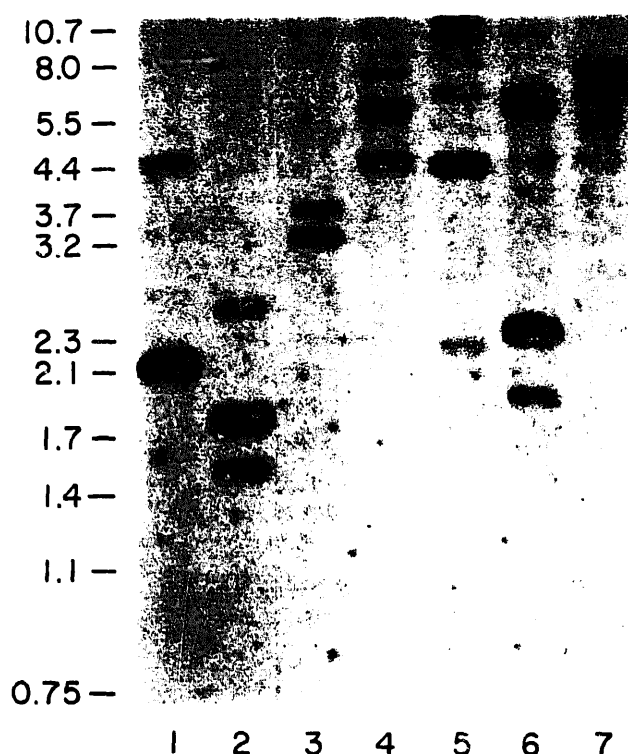


Fig. 3. Southern blot analysis of genomic DNA probed with a ^{32}P -labeled Cyp1 cDNA sequence. Genomic DNA was prepared from *C. albicans* blastospores using protocols similar to those developed for *S. cerevisiae* (Abraham et al., 1984; Sherman et al., 1986). Restriction endonuclease-digested DNA was subjected to electrophoresis through 0.8% agarose, followed by transfer to nitrocellulose. Blots were probed with a ^{32}P -labeled DNA fragment as described in Fig. 2, using identical hybridiza-

tion conditions. Filters were washed at 65°C in 0.3 × SCP/0.1% SDS, and autoradiographed. Lanes: 1, *Eco*RI; 2, *Hind*III; 3, *Bgl*II; 4, *Eco*RV; 5, *Pvu*II; 6, *Nco*I; 7, *Bam*HI. None of these enzymes, except *Nco*I, cuts within the cDNA sequence. There is a single *Nco*I site in the cloned *CYP1* cDNA sequence. Each lane contained 10 µg of DNA.

that they share a high degree of sequence similarity, with the exception of the N- and C-terminal 20 or 30 aa (Fig. 4). The predicted *CYP1* gene product exhibits approximately 81 and 53% aa homology with Cyps from *S. cerevisiae* (Haendler et al., 1989; Koser et al., 1990). In addition, the *CYP1* gene product shares 72, 68 and 47% sequence homology with Cyps from *N. crassa* (Tropschug et al., 1988), human T-cells (Haendler et al., 1987), and *D. melanogaster* (Schneuwly et al., 1989), respectively. Three consensus sequences, NGTGGKSIYG, LSMANAGPNTN, GSQFF and WLDGKHVVFG (Haendler et al., 1989) are common to all eukaryotic Cyps examined to date, including the *C. albicans* enzyme. The high degree of conservation of these peptide sequences in Cyps suggests that they may play some role in either CsA binding or in PPIase activity.

It is noteworthy that of the four cysteines conserved among mammalian Cyps (Bergsma and Sylvester, 1990; Danielson et al., 1988; Fischer et al., 1989b; Haendler et al., 1987; Harding et al., 1986), one of which is thought to play a role in a PPIase catalytic mechanism (Fischer et al., 1989a), none are conserved in number and position within the *C. albicans* sequence.

	1	42
CA-Cyp	MSTVIFD VSADGQKL.....GKITFKLY DDVVPKTAEN FRALC.TGEX	
SC-Cyp1	Q-----E-----PI.....-RVV-----N-I-----	
SC-Cyp2	M KFSGLWCWLL LFSLVNVIAS DVGELIDQDD EVITQK-F- IEHGEE-V.....-R-VIG-- GK-C-----K- -YK-S.-TN	
NC-Cyp	MFGPRHFSVL KTTGSLVSST FSSSLKPTAT FSCARAFSQT SSI--K-F- LEWE-PV-GP NNKPTSEIKA QS-R-N-T- -----R- -KE---.---Q	
DM-Cyp	MK SLLNRIILCS AFLAVASGLS FTVT-RI-M- -KHNNKPV.....-R---G-F GKLA---VA- --HI-LR-IN	
HS-Cyp	MVNP--F-- IAVN-EP-.....-RVS-E-F A-K-----G- ----S----	
	43	136
CA-Cyp	G.FGYKGSIF HRVIPQFMLQ GGDFTNFNGT GGKSIYGTKF ADEN..FVKR HDRPGLLSMA NAGPNTNGSQ FFITTV.PCP WLDGKHVVFG FVTDG..LDI	
SC-Cyp1	---A--P- ----D-----AG-----G- P---.-K-H -----V---Y--	
SC-Cyp2	SKK-FI--T- ----N--V- ----DGT-V -----DT- P---.-TLK --K-R-----R-KD-----TEEAS -----Q-V---M-V	
NC-Cyp	---S--I--E-----RG-----E- ----.-A-K -V-----V---.-TS ----R-----A-DESMV	
DM-Cyp	..TS-V--R- --VDR-LV- --IV-GD-- -SI---DY- P--DKA-AVE -N---Y-G-- -R--D---C- -V---.GAK -----T---K-LE--M-	
HS-Cyp	---C--I--G--C- ----RH-----E- E---.-ILK -TG--I-----C-A.KTE -----K-KE--M-	
	137	162
CA-Cyp	VKKIESFGSG SGAT..SKKI V.IEESGQL.....	
SC-Cyp1	---V--L--P ----.KAR- -VAK--E-.....	
SC-Cyp2	-NY-QHVS RD ANDK..PLEA -K-AKC-EWT PELSS.....	
NC-Cyp	--AL-AT--S ---IRY---P T.-VDC-A..	
DM-Cyp	IYA--DVKTD TDD.FPVEPV --SNC-EIP TEQFEFYPDD FNILGWIKAA GLPVTSSFCV LLIFHYFFRQ LNMVY	
HS-Cyp	-EAM-R---R N-K-----T.-ADC---E.....	

Fig. 4. Comparison of the aa sequences of Cyps from various sources. CA-Cyp: *C. albicans* Cyp (deduced *CYP1* gene product); SC-Cyp1: *S. cerevisiae* cytosolic Cyp (Haendler et al., 1989); SC-Cyp2: a second *S. cerevisiae* Cyp (deduced gene product) (Koser et al., 1990); NC-Cyp: *N. crassa* Cyp (Tropschug et al., 1988); DM-Cyp: *D. melanogaster* Cyp (Schneuwly et al., 1989); HS-Cyp: human T-cell Cyp (Haendler et al., 1987). Numbering of the aa starts with the Met of the CA-Cyp sequence. Dashes indicate aa identity with the *C. albicans* sequence, periods indicate sequence gaps introduced to maximize sequence homology. The cysteines are underlined in the human and *C. albicans* sequences.

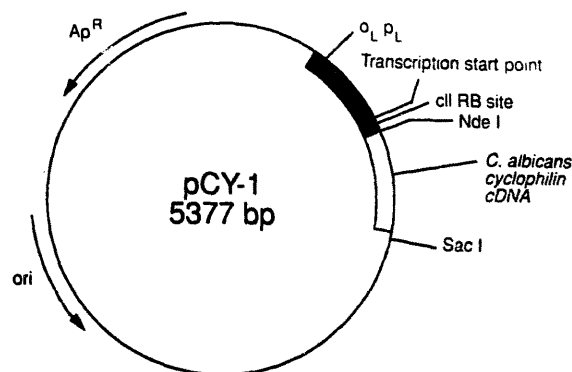
(d) Expression of *C. albicans* Cyp cDNA in *Escherichia coli*

Inasmuch as the porcine and *S. cerevisiae* proteins have been shown to be peptidyl-prolyl *cis-trans* isomerases (Fischer et al., 1989b; Haendler et al., 1989; Takahashi et al., 1989), we wanted to determine whether the *C. albicans* *CYP1* recombinant gene product exhibits a similar enzymatic activity. To this end the cDNA was engineered for expression in *E. coli*. The expression vector system is shown in Fig. 5A. Fig. 5B shows that an 18-kDa protein was produced from heat-induced *E. coli* AR-58 cells containing the Cyp expression plasmid pCY-1 (lanes 7,8,9). The 18-kDa protein was not expressed in cultures containing the control plasmid pMG-1 alone (lanes 2–5), nor was it expressed in uninduced cultures containing pCY-1 (lane 6). Most of the 18-kDa protein is soluble after bacterial lysis (lane 8). Laser densitometry suggests that the expressed Cyp accounts for at least 30% of total cell protein (data not shown). Despite this large accumulation of heterologous protein, the bacterial host cells did not exhibit a noticeable change in growth characteristics when compared with cells transformed with the control plasmid, pMG-1. The 10-kDa protein expressed in cells containing pMG-1 (lane 3) is derived from a sequence which was deleted during the construction of pCY-1. To confirm the identity of the product, the N-terminal aa sequence of the 18-kDa protein was determined following SDS-PA gel purification (Matsudaira, 1987). The N-terminal sequence (STVYFDVSADGQKLG) was found to be identical to aa residues 2–16 of the deduced Cyp sequence, with the N-terminal methionine apparently having been processed in *E. coli*.

(e) PPIase activity of the expressed Cyp

The PPIase activity in the soluble fraction of an *E. coli* extract containing the expressed *C. albicans* Cyp is presented in Fig. 6. The net rate constant for the catalytic isomerization per mg of protein is enhanced approximately 140-fold with the extract containing the expressed *C. albicans* protein ($170 \text{ sec}^{-1} \text{ mg}^{-1}$) as compared to that present in the *E. coli* host ($1.2 \text{ sec}^{-1} \text{ mg}^{-1}$), demonstrating that the recombinant *C. albicans* Cyp is active as a PPIase. The catalytic efficiency of the soluble fraction containing the *C. albicans* PPIase (approx. $170 \text{ sec}^{-1} \text{ mg}^{-1}$) is comparable to that determined with *E. coli* extracts possessing recombinant human Cyp (approx. $180 \text{ sec}^{-1} \text{ mg}^{-1}$) which has been expressed at about 30% of the soluble protein (Bergsma and Levy, 1990). Furthermore, the PPIase activity of the recombinant *C. albicans* Cyp was completely inhibited by 100 nM CsA, while the endogenous *E. coli* activity was not affected (Fig. 6). These results demonstrate that expression of *CYP1* produces an active PPIase which is sensitive to the immunosuppressive agent CsA. These

A.



B.

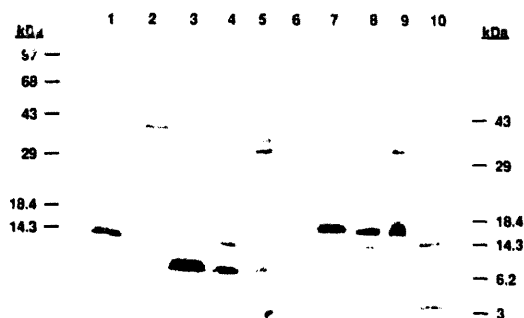


Fig. 5. Expression of *C. albicans* *CYP1* in *E. coli*. (A) *E. coli* expression vector pCY-1. A full length *C. albicans* cyclophilin cDNA fragment was engineered by PCR to contain an *Nde*I site and a *Sac*I site at the 5' and 3' ends, respectively. This fragment was subcloned into the *E. coli* expression vector pMG-1 (a derivative of pAS-1; Shatzman and Rosenb erg, 1987), downstream of the *cII* ribosome-binding site and transcription initiation site of the bacteriophage λ promoter ($o_L P_L$). The resulting plasmid (pCY-1), as well as pMG-1, were each transfected into *E. coli* AR-58 cells. These host *E. coli* cells supply the temperature-sensitive CI857 repressor. The Ap^R gene and plasmid origin of replication are denoted by arrows. Solid box, bacteriophage promoter; open box, *C. albicans* *CYP1* cDNA. (B) SDS-PA analysis of the expression products of *E. coli* AR-58 cells transformed with either pMG-1 (lanes 2–5) or pCY-1 (lanes 6–9). Cultures were grown at 30°C in Luria broth (including 100 μg Ap per ml) to an optical density (550 nm) of 0.7, and subsequently transferred to 42°C for an additional 3 h to induce expression. Following centrifugation, cell pellets were resuspended in 20 ml of 50 mM Tris-HCl pH 8.0/2 mM EDTA/0.1 mM DTT/5% glycerol/10 μg lysozyme per ml. The suspensions were incubated for 30 min at 4°C, sonicated, and centrifuged at 12000 $\times g$. Aliquots of supernatants were subjected to SDS-PAGE (Laemmli, 1970) using separating and stacking gels containing 20 and 5% acrylamide, respectively. Shown are the uninduced cultures (lanes 2 and 6), heat induced cultures (lanes 3 and 7), lysate supernatants (lanes 4 and 8), and lysate pellets (lanes 5 and 9). Each lysate supernatant lane represents 0.04% of the supernatant fraction whereas each lysate pellet lane represents 0.5% of the pellet fraction. M_r markers are shown in lanes 1 and 10. The gel was stained with Coomassie blue. The 14-kDa protein in the supernatants and pellets of both cultures corresponds to exogenously added lysozyme.

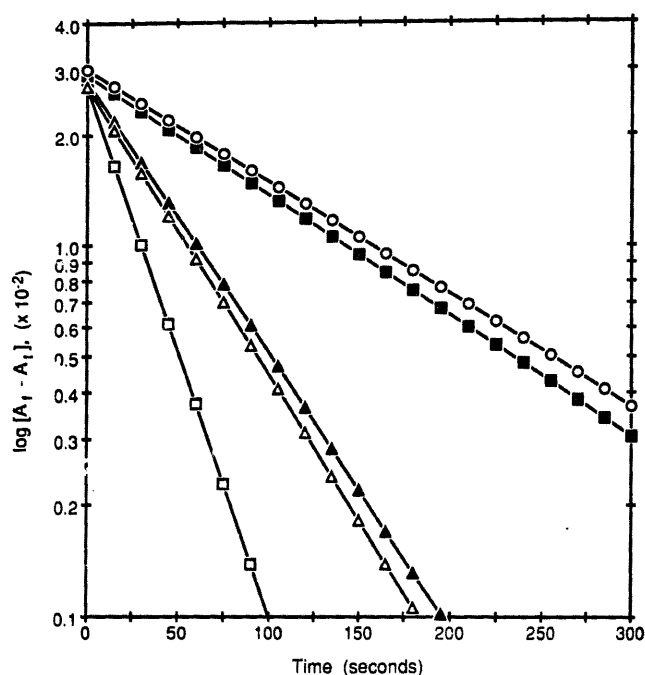


Fig. 6. PPIase activity of *Candida albicans* Cyp and inhibition by CsA. PPIase activity in the extracts of heat-induced *E. coli* cultures was determined using a synthetic peptide, succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Sigma Chemical Co., St. Louis, MO), as substrate in a two-step reaction coupled to chymotrypsin (bovine pancreatic α -chymotrypsin, Type II, EC 3.4.21.1, Sigma Chemical Co.) as described by Fischer (1984). Protein concentrations were estimated by the method of Bradford (1976). CsA was obtained from Sandoz, Inc., East Hanover, NJ. All assays were performed in 1 ml at 10°C in 35 mM HEPES, pH 7.8 with 25 μ M peptide substrate and monitored at 390 nm with a Perkin-Elmer Lambda 4C UV/VIS spectrophotometer. Assays were initiated by the addition of α -chymotrypsin (100 μ g per assay) and the absorbance measured at timed intervals for 5 to 10 min. The data were analyzed using the equation $A_t = A_f - C \cdot e^{-kt}$, where A_t is the absorbance at a given time (t), A_f is the final absorbance at infinite time, C is the absorbance range of the slow phase corresponding to the *cis* to *trans* isomerization of the peptide substrate, and k is the first-order rate constant for the alanyl-prolyl *cis-trans* isomerization (Fischer et al., 1984). Fitting of data to this equation was accomplished with a nonlinear regression procedure using the Marquardt algorithm (1963) in the SAS statistical analysis software package (SAS Institute, Inc., Cary, NC). The data are plotted as the log ($A_f - A_t$) versus time. The slope of each curve represents the rate constant (k). The rate constant for the spontaneous reaction in the absence of extract (O) was determined to be $0.0070 \pm 0.0004 \text{ sec}^{-1}$. With the *E. coli* extract (9 μ g protein) containing the plasmid pMG-1 (no expressed CYP-I gene product) the rate constants in the absence (Δ) and presence (□) of 100 nM CsA were $0.0175 \pm 0.0012 \text{ sec}^{-1}$ and $0.0182 \pm 0.0009 \text{ sec}^{-1}$, respectively. In the presence of a significantly lower amount of *E. coli* extract (0.15 μ g protein) containing the expressed CYP1 protein (plasmid pCY1) the *cis* $\rightarrow$ *trans* isomerization rate was $0.0324 \pm 0.0012 \text{ sec}^{-1}$ (□), which decreased to $0.0075 \pm 0.0005 \text{ sec}^{-1}$ in the presence of 100 nM CsA (■). In experiments to determine the inhibition by CsA, inhibitor in ethanol was added to the incubation mixture prior to the initiation of the reaction. The final concentration of ethanol did not exceed 1%, which does not effect enzymatic activity.

characteristics are identical to those reported for the porcine Cyp (Fischer et al., 1989b; Takahashi et al., 1989).

It has been proposed that the catalytic mechanism of the porcine PPIase (98% homologous to human T-cell Cyp)

proceeds through an enzyme-bound tetrahedral intermediate involving a hemithio-orthoamide (Fischer et al., 1989a) derived from a single cysteine residue of the enzyme. For homologous enzymes catalyzing the same reaction, such as mammalian and *C. albicans* Cyps, we would anticipate that the catalytic mechanisms would be related. *C. albicans* Cyp contains two cysteines (Cys 38 and Cys 117; Fig. 4), neither of which is in an identical position to any of the four cysteines in the mammalian sequences. Since *C. albicans* and human T-cell Cyps would appear to demonstrate equivalent levels of PPIase catalysis, the lack of conservation of cysteines in the sequence of these proteins would seem incongruous with a catalytic mechanism involving an enzyme-bound hemithio-orthoamide, and would better support a PPIase mechanism involving catalytic distortion (Harrison and Stein, 1990). This observation is consistent with a very recent report examining the catalytic role of cysteines in human recombinant Cyp (Liu et al., 1990). In that study, when cysteines were changed individually to alanines, the resulting mutated proteins each exhibited a CsA-binding affinity and a PPIase activity comparable to wild-type Cyp.

(f) Conclusions

We have cloned both a genomic and a cDNA sequence encoding a protein from *C. albicans* which shares a high level of homology with human Cyp. We designate this gene *CYP1*. The cDNA, when expressed in *E. coli*, yields a soluble 18-kDa protein which exhibits PPIase activity. Unlike the cytosolic *S. cerevisiae* enzyme (Haendler et al., 1989), the level of enzymatic activity is equivalent to that of mammalian Cyps.

Furthermore, this PPIase activity is inhibited by CsA, indicating that the *C. albicans* *CYP1* gene product is functionally similar to the porcine (Fischer et al., 1989b; Takahashi et al., 1989) and human (Bergsma and Levy, 1990) enzymes. Genomic Southern blot data are consistent with the existence of two or more Cyp-related sequences in *C. albicans*.

Although the primary role of the *CYP1* gene product is presumed to be related to protein folding, its precise molecular function remains to be elucidated. For example, it will be important to determine whether the *CYP1* gene product is essential for cell viability. Assessment of the potential pharmacological significance of the *CYP1* gene product, as well as other PPIases, as targets for the development of antifungal agents will require further molecular genetic analysis.

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