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Overexpression, purification, and characterization of yeast cyclophilins A and B

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Abstract

Two isoforms of yeast cyclophilins, yCyPA and yCyPB, have been subcloned, expressed in *Escherichia coli*, and purified to homogeneity. The full-length (163-amino acid) yeast CyPA was easily expressed and purified; however, only a genetically truncated, 186-residue form of yCyPB lacking a putative 20-amino acid signal sequence could be purified. Each yeast cyclophilin isoform is a peptidyl-prolyl isomerase, inhibitable by the immunosuppressive drug CsA (IC_{50}'s of 40 ± 8 nM and 101 ± 14 nM at 18 nM concentrations of yCyPA and yCyPB, respectively). Polyclonal antibodies raised against recombinant yCyPA detected native yCyPA in yeast cell extracts by both immunoprecipitation and Western blot analysis. However, polyclonal antibodies raised against recombinant yCyPB detected no native yCyPB in yeast cell extracts by Western blot analysis; small amounts of yCyPB were found in the culture broth, suggesting secretion extracellularly of this isoform. Northern analysis indicated that both *yCyPA* mRNA and *yCyPB* mRNA (at a much lower level) were detectable in cell-free extracts. Characterization of the yeast cyclophilin proteins demonstrated that their catalytic properties and sensitivity to CsA parallel those of the human cyclophilins.

Keywords: cyclophilin; cyclosporin A; peptidyl-prolyl isomerase

The CyP class of proteins was initially characterized in higher eukaryotes (e.g., human and bovine spleen) on the basis of high binding affinity toward the immunosuppressive drug CsA (Handschumacher et al., 1984; Harding et al., 1986). CsA blocks T-cell activation by inhibiting the activation of cytokine gene transcription (Elliott et al., 1984; Kronke et al., 1984). The cyclophilins are widely distributed and abundant intracellular proteins; CyP gene sequences have been found in yeast (Haendler et al., 1989; Dietmeier & Tropschug, 1990; Koser et al., 1990a,b), *Neurospora* (Tropschug et al., 1988), various plants (Gasser et al., 1990), and bacteria (Liu & Walsh, 1990; Hayano et al., 1991). Several possibilities for the physiological role of cyclophilins and the pharmacological mechanism of CsA have been suggested based on the demonstration of PPIase catalytic activity of purified cyclophilins, first in pig (Fischer et al., 1989; Takahashi et al., 1989), then in yeast (Haendler et al., 1989; Koser et al., 1991), human (Liu et al., 1990), *Escherichia coli* (Liu & Walsh, 1990), and more recently other species (Hasel et al., 1991; Hayano et al., 1991; Spik et al., 1991). One hypothesis is that cyclophilins play a role in protein folding via isomerization of the cis-trans X-Pro bond. Blockade by CsA of this activity toward a specific protein substrate, e.g., in the transcriptional activation cascade, could be a mechanism of T-cell-directed immunosuppression, but recently attention has been focused on the CsA-CyP complex as the active species in the inhibition of T-cell activation (Tropschug et al., 1989). This drug-immunophilin complex, as well as that of another immunosuppressant FK506 and its binding protein FKBP, inhibits in vitro the Ser/Thr phosphatase activity of the Ca^{2+}/calmodulin-dependent protein, calcineurin (Liu et al., 1991b).

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Abbreviations: CyP, cyclophilin; CsA, cyclosporin A; PPIase, peptidyl-prolyl isomerase; FKBP, FK506 binding protein; hCyPB, human cyclophilin B; yCyPA, yeast cyclophilin A; yCyPB, yeast cyclophilin B; hCyPA, human cyclophilin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEAE-Sepharose, diethylaminoethyl-Sepharose; TCA, trichloroacetic acid; AAPF, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; PCR, polymerase chain reaction; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); IC_{50}, half-maximal inhibitory concentration; FCA, Freund's complete adjuvant; IFA, incomplete Freund's adjuvant; IgG, immunoglobulin G; PBS, phosphate-buffered saline, pH 7.2; TE, 10 mM Tris-HCl/1 mM EDTA (pH 8.0).

Although initial studies indicated that bovine CyP is a cytosolic protein (Handschumacher et al., 1984), a *Neurospora crassa* CyP has been demonstrated to be associated with the mitochondria (Tropschug et al., 1988). In several species where multiple CyP genes have been identified, at least one isoform contains a hydrophobic N-terminal extension. We have recently reported a periplasmic *E. coli* CyP (Liu & Walsh, 1990), and a second human CyP gene (hCyPB) with an N-terminal signal sequence (Price et al., 1991). Yet a third human CyP gene, containing an N-terminal extension, has also been identified (Bergsma et al., 1991), as well as a homolog with both N- and C-terminal extensions in the murine AC-6 cell line (Friedman & Weissman, 1991). In addition to the yeast CyP (yCyPA) isolated and partially characterized by Haendler and coworkers (Haendler et al., 1989; Dietmeier & Tropschug, 1990), a second yeast gene sequence, termed yCyPB, with an N-terminal extension to the coding region, has recently been reported (Koser et al., 1990a). NinaA, a *Drosophila* CyP-like protein, is a tissue-specific integral membrane protein required for the folding of a subset of rhodopsins (Stamnes et al., 1991). It has a signal sequence and appears to be a CyPB isoform (Schneuwly et al., 1989; Shieh et al., 1989). Given the array of B-type cyclophilins containing N-terminal extensions (Caroni et al., 1991; Hasel et al., 1991; Spik et al., 1991), the question arises whether multiple CyP genes in eukaryotes may indicate distinct cellular compartmentalization and perhaps specific functions for different CyP isoforms.

To assess and compare properties of the two yeast CyP isoforms such as PPIase catalytic activity, susceptibility to CsA, and cellular compartmentalization, we report the overexpression of both yCyPA and yCyPB in *E. coli*. Protein purification, characterization, and detection of each yCyP by northern blotting and immunochemical techniques in yeast cell extracts were performed.

Results

Expression of yCyPA and yCyPB in E. coli: Purification and characterization

To compare the catalytic properties of the proteins encoded by the two yeast genes *yCyPA* and *yCyPB*, assess the sensitivity to inhibition by CsA, and obtain protein for immunochemical studies, *E. coli* overexpression vectors were constructed as described in the Materials and methods, and the proteins were purified to homogeneity. Recombinant yCyPA was purified as reported previously for hCyPA (Liu et al., 1990), with a yield of 58 mg of pure protein from 1 L of *E. coli* fermentation. We previously encountered difficulty with the expression and purification of full-length hCyPB but were able to obtain good results using a construct in which the N-terminal 24 residues comprising the signal sequence had been deleted (Walsh et al., unpubl.). With that in mind, we similarly expressed a yCyPB lacking the first 20 putative signal sequence residues. All studies reported here were performed on the genetically truncated form of yCyPB. Yeast CyPB displayed somewhat different properties from yCyPA on column chromatography, but by modifying the purification procedure a yield of 10.0 mg of pure genetically truncated yCyPB was obtained from 1 L of culture. Yeast CyPB has a predicted 206 amino acids (aa), versus 163 aa as predicted for yCyPA, corresponding to approximately 56% homology (Fig. 1). As shown in Figure 2, recombinant yeast cyclophilins expressed in *E. coli* yield yCyPA with a molecular weight of 17 kDa (lane 2) and yCyPB with a mobility of 20.5 kDa (lane 3). The N-terminal sequence analyses of yCyPB (genetically truncated) yielded SDVGELIDQDDEVIT and of yCyPA, SQVYFDVEADGQPIGRVVFKLYND, which are in agreement with those predicted for each from their respective gene sequences. Isoelectric focusing of yCyPA yielded only one isoform with a pI of 7.0 in contrast to the previously re-

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                                        ↓
yCyPB : MKFSGLWCWLLLFLSVNVIASDVGELI.........DQDDEVITQKVFFDIEHGEEKVGR
yCyPA : -S.....................................Q-Y--V-ADGQPI--
hCyPA : -.....................................VNPT-----AVDG-PL--
hCyPB : --.........VL-AAAL--GS-FF-LlpgpsaadeKKKGPKV-V--Y--LRI-D-D---

yCyPB : IVIGLYGKVCPKTAKNFYKLSTTTNSKKGFIGSTFHRVIPNFMVQGGDFTDGTGVGGKSI
yCyPA : V-FK--NDIV----E--RA-.C-GEKGF-YA--P------D--L------A-N-T-----
hCyPA : VSFE-FADKV----E--RA--.-GEKGF-YK--C---I--G--C------RHN-T-----
hCyPB : VIF--F--TV---VD--VA-.A-GEKGF-YKN-K-----KD--I------R-D-T-----

yCyPB : YGDTFPDENFTLKHDRKGRLSMANRGKDTNGSQFFITTTEEASWLDGKHVVFGQVVDGMD
yCyPA : --GK------KKH---P-L-----A-PN--------.--VPCP----------E----Y-
hCyPA : --EK-E----I---TGP-I-----A-PN--------.C-AKTE----------K-KE--N
hCyPB : --ER------K---YGP-WV----A-----------VKT-.----------K-LE--E

yCyPB : VVNYIQHVSRDANDKPLEAVKIAKCGEWTPE....LSS@
yCyPA : I-KKVESL.....GS-SG-T-ARIVVAKSG-....-@..
hCyPA : I-EAMERFG.SR-G-TSKKIT--D--QLE.........
hCyPB : --RKVESTKT-SR----KD-I--D--KIEV-kpfaIAKE
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Fig. 1. Sequence comparisons of hCyPA and hCyPB and yCyPA to yCyPB. Underlined residues indicate N-terminal signal sequences found in B cyclophilins. The arrow denotes the site chosen for signal sequence truncation before subcloning into *E. coli* overexpression vector. yCyPB, *S. cerevisiae* secretory CyP (Koser et al., 1990a); hCyPA, human cytosolic CyP (Haendler et al., 1987); hCyPB, human secretory CyP (Price et al., 1991); yCyPA, *S. cerevisiae* cytosolic CyP (Haendler et al., 1989). Dashes indicate identity with yCyPB residues; capital letters indicate conservative changes; periods indicate gaps opened to maximize sequence homology.

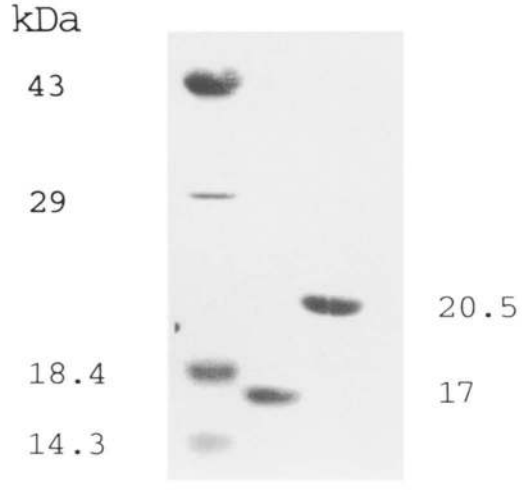

Fig. 2. SDS-PAGE (15%T) analysis of purified recombinant *S. cerevisiae* cyclophilins A and B, stained with Coomassie blue R250. Lanes: 1, molecular weight standards; 2, yCyPA; 3, yCyPB.

ported pI of 6.45 (Haendler et al., 1989). However, yCyPB (homogeneous on SDS-PAGE analysis) was resolved into two isoelectric forms of approximately equal abundance with pIs of 4.9 and 5.0 (data not shown).

Catalytic properties and susceptibility to inhibition by CsA of recombinant yCyPA and yCyPB

Using the standard chromogenic substrate AAPF in the chymotrypsin-coupled PPIase assay in which the cis-trans isomerization of the A-P bond is monitored at 10 °C (Fischer et al., 1984a,b), the pure yCyPA and yCyPB proteins were assayed side by side at equivalent concentrations. Each is an efficient catalyst, as noted in Table 1, with yCyPA (k_{cat}/K_m of 1.52×10^7 M^{-1} s^{-1}) being 2.6-fold more efficient than yCyPB (k_{cat}/K_m of 5.77×10^6 M^{-1} s^{-1}) with this substrate. Koser et al. (1991) have recently reported values of 1.1×10^7 M^{-1} s^{-1} for a yCyPB truncated slightly differently. A general characteristic of the eukaryotic CyP family, in contrast to the prokaryotic homologs (Liu et al., 1991a), is sensitivity to inhibition of the PPIase activity by the immunosuppressive drug CsA. Both yeast cyclophilins when assayed at enzyme concentrations of 18 nM showed half-maximal sensitivity in the 40–100 nM range as depicted in Table 1, yCyPA being 2.5-fold more sensitive to inhibition by CsA. These values are well within the range of sensitivity of other eukaryotic cyclophilins to CsA (Liu et al., 1990, 1991a; Price et al., 1991).

Table 1. *Kinetic and IC_{50} determinations for the recombinant human and yeast cyclophilins purified from E. coli overexpression systems*[a]

CyP	k_{cat}/K_m (M^{-1} s^{-1})	CsA IC_{50} (nM)
hCyPA (6.7 nM)	1.6×10^7	25 ± 5
hCyPB (6.9 nM)	6.3×10^6	84 ± 12
yCyPA (18 nM)	1.52×10^7	40 ± 8
yCyPB (18 nM)	5.77×10^6	101 ± 14

[a] k_{cat}/K_m and IC_{50}'s were determined as described in the Materials and methods.

Immunochemical studies of cyclophilins

Antibodies raised against pure recombinant yCyPA and yCyPB recognized yCyPA and yCyPB, respectively, with slight cross-reactivity, by Western analysis of the purified recombinant proteins on SDS-PAGE as shown in Figure 3. Immunoprecipitation studies of yeast cell extracts were also performed with the anti-yCyPA antibody to determine if any proteins were associated with yCyPA. Only yCyPA was detected under the conditions used (data not shown).

Western blot analysis of total yeast extract, with the same yCyPA antibody, likewise yielded only a band corresponding in size to pure recombinant yCyPA. To increase the sensitivity of detection, analysis was also performed using ^{125}I-tagged secondary antibody. Strong bands corresponding to yCyPA but not yCyPB were detected following autoradiography. When total yeast extract was screened with the anti-yCyPB antibody, no bands were visible upon autoradiography, although a strong signal was seen for pure recombinant yCyPB. The

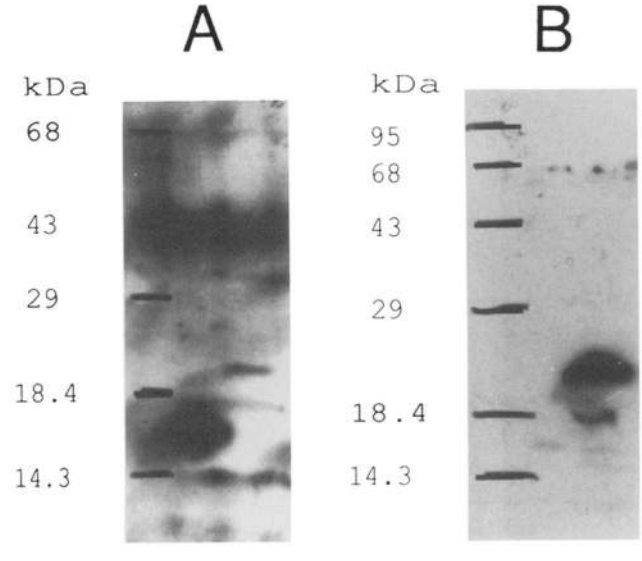

Fig. 3. Autoradiographs of Western analysis of *S. cerevisiae* cyclophilins A and B. Lanes: 1, molecular weight standards; 2, pure recombinant yCyPA (500 ng); 3, pure recombinant yCyPB (500 ng). Samples were electrophoresed on an SDS-acrylamide (15%T) gel, electroblotted to nitrocellulose (150 mA, 8 h), and (**A**) screened with polyclonal rabbit antibodies raised against recombinant yCyPA or (**B**) screened with polyclonal rabbit antibodies raised against recombinant yCyPB. ^{125}I-tagged donkey antirabbit IgG secondary antibodies (20 μCi; Amersham) were used in both experiments.

possibility of yCyPB being secreted extracellularly was then addressed by performing Western analysis, using the anti-yCyPB antibody, on proteins in the culture broth of yeast cells following DEAE-Sepharose adsorption, concentration, and TCA precipitation. A single faint band corresponding to yCyPB was visualized following overnight autoradiography (Fig. 4).

Detection of yCyPA and yCyPB mRNA by Northern analysis

To confirm the expression of yCyPA and yCyPB protein seen in Western blots of yeast extract and culture broth, respectively, and to determine to what degree yCyPB is expressed, total RNA extracted from yeast was analyzed with either *yCyPA* or *yCyPB* DNA under stringent hybridization conditions. As controls, total RNA samples from single and double knockout *Saccharomyces cerevisiae* strains (provided by M. Brennan, NIMH) were probed concomitantly and gave the expected signals. Upon analysis with the *yCyPA* probe, one band corresponding to an ~960-nucleotide (nt) mRNA was observed in both normal and heat-shocked samples after overnight autoradiography. Probing with *yCyPB* DNA under the same conditions revealed a weak signal corresponding to an ~1,020-nt mRNA in normal and heat-shock samples after a 48-h exposure (data not shown).

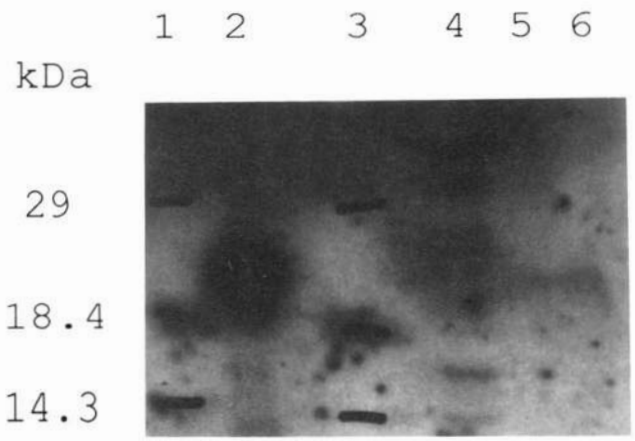

Fig. 4. Autoradiograph of Western analysis of *S. cerevisiae* cell pellet and culture broth. Lanes: 1, molecular weight standards; 2, pure recombinant yCyPB (500 ng); 3, molecular weight standards; 4, *S. cerevisiae* total cell extract, prepared by microcentrifugation of 1.5 mL of mid-log phase YEPD culture (OD_{600} ~ 2), washing the pellet with 200 μL TE, lysing cells by vortexing in 200 μL SDS Laemmli buffer with 200 μL glass beads, and boiling for 5 min; 5, dissolved TCA precipitate of 750 mM NaCl wash of *S. cerevisiae* culture broth; 6, dissolved TCA precipitate of 500 mM NaCl wash of *S. cerevisiae* culture broth, prepared by adsorption of 1 L *S. cerevisiae* culture broth to 250 mL DEAE-Sepharose (20 mM Tris-HCl, pH 8.0) overnight, followed by successive washes of 0, 250, 500, 750, and 1,000 mM NaCl in 20 mM Tris-HCl, pH 8.0. Protein in concentrated washes was precipitated with 8% TCA, redissolved in SDS Laemmli buffer, and boiled for 5 min. Samples were subjected to 15%T SDS-PAGE and electroblotted to nitrocellulose (150 mA, 8 h). Western analysis was performed using polyclonal rabbit antibodies raised against recombinant yCyPB and 20 μCi ^{125}I-tagged donkey antirabbit IgG secondary antibodies (Amersham).

Discussion

We report in this study the purifications to homogeneity and characterizations of yCyPA (163 aa) and yCyPB (186-aa fragment, with the first 20 aa deleted) from cloning by PCR and expression in *E. coli*, and localization of the native proteins by Western analysis in *S. cerevisiae*. The availability of the two recombinant yCyP isoforms allows direct comparison of these two homologous proteins. They are not only structurally similar to their human counterparts (Price et al., 1991) but also parallel them in PPIase activity and sensitivity to inhibition by CsA (Table 1).

It has been recently determined that the full-length human CyPB homolog is cleaved in *E. coli* between the 25th and 26th residues to remove the signal sequence (Price et al., 1991). Because reasonable quantities of the full-length yCyPB expressed in *E. coli* could not initially be obtained, it was assumed that yeast CyPB would be processed in a similar manner. Accordingly, the first 20 residues, corresponding to the putative signal sequence (Koser et al., 1990a), were removed, resulting in a genetically truncated construct that gave a yield of 10 mg of pure yCyPB protein from 1 L of *E. coli* cell growth.

The presence of N-terminal signal sequences in the 206-aa yCyPB and 208-aa hCyPB suggests that the CyPB subfamily is directed to subcellular locations other than the cytoplasm. In contrast, the 163-aa yCyPA and 165-aa hCyPA are thought to be cytosolic. Sequence alignments of yeast and human CyPB (Fig. 1) show only 32% homology in the signal sequence region (33 aa), but 57% homology over the next 165 aa, thought to be the core CyP domain. In addition, the CyP homolog ninaA, in *Drosophila*, has an N-terminal signal sequence and is a representative of the CyPB subfamily. It is localized as an integral membrane protein in the endoplasmic reticulum of photoreceptor cells and has been suggested to be involved in the folding of a subset of rhodopsin molecules (Schneuwly et al., 1989; Shieh et al., 1989; Stamnes et al., 1991). Epitope tagging studies of hCyPB and hCyPA with the c-*myc* epitope followed by transfection into HeLa cells show that, whereas hCyPA appears equally in the nucleus and cytoplasm, hCyPB is distributed in the endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicles (McKeon et al., unpubl.); similarly mouse CyPB has recently been localized to microsomal fractions via a mouse CyPB-specific antibody (Hasel et al., 1991). Tanida et al. (1991) have also recently reported the secretion of overexpressed recombinant yCyPB from *S. cerevisiae* cells.

In order to determine the localizations of yCyPA and yCyPB, we obtained polyclonal antibodies to both recombinant proteins. However, Western analyses with both antibodies, as well as immunoprecipitations with

anti-yCyPA antibody under native and denaturing conditions of total yeast extracts, consistently showed a signal for native yCyPA but not for yCyPB. To verify this result, single and double CyP knockout strains (M. Brennan) were tested as controls. A signal for yCyPA was observed in all yCyPA$^+$ strains, but yCyPB could not be detected in cell pellet extracts of any strain tested, ruling out a subcellular or membrane-associated location for yCyPB. However, Northern blot analyses, using the *yCyPA* and *yCyPB* DNA as probes against total yeast RNA and RNA from knockout strains (M. Brennan) as controls, elicited signals for the *yCyPA* mRNA corresponding to a 960-nt transcript and for the *yCyPB* mRNA at 1,020 nt. Detection of a faint yCyPB protein signal in the culture broth upon Western analysis indicates that the *yCyPB* mRNA is translated, albeit at very low levels. Furthermore, if the protein is modified, e.g., by glycosylation, the effective titer of polyclonal antibodies recognizing epitopes on the protein may be reduced, making detection more difficult. In any event, the extracellular location of yCyPB indicates that it has little or no role in the sensitivity of yeast to CsA. This is borne out by recent findings (Koser et al., 1991; Tanida et al., 1991) that null mutations at the yCyPB locus fail to generate resistance to CsA.

Distinct intracellular compartmentalization for CyPA versus CyPB isoforms in both human and mouse suggests that the two classes may interact with distinct subsets of substrate and partner proteins. Both pure yCyPA and yCyPB (and also pure hCyPA and hCyPB [Liu et al., 1990; Price et al., 1991]) have similar catalytic efficiencies in the tetrapeptide-based PPIase assay (Table 1). Whereas the cytosolic CyPAs (yeast and human) could be involved in folding proteins destined for the cytosol or nucleus (e.g., transcription factor components), endoplasmic reticulum and Golgi apparatus-affiliated CyPB species may refold proteins extruded into the endoplasmic reticulum lumen, destined for secretory pathways. As yet only in *Drosophila* have specific protein substrates, members of a subset of rhodopsin molecules, been identified for the endoplasmic reticulum-bound CyP ninaA (Schneuwly et al., 1989; Shieh et al., 1989; Stamnes et al., 1991). It is unknown whether additional cyclophilins will be found in the intracellular membrane system of yeast and higher eukaryotes.

CsA is known to have a multiplicity of effects on human T-cell and mast cell function, including blockage of cytokine gene transcriptional activation (CyPA involvement?) and of exocytosis and vesicle degranulation (CyPB involvement?). It is not at present clear which effects are mediated by which subfamily of cyclophilins. Furthermore, the complexity of the pathways affected by CsA creates difficulties in the identification and study, by purely biochemical means, of cyclophilins specifically associated with various effects.

Due to the high homology between the yeast and human cyclophilins, yeast as a eukaryotic system offers potential advantages as a genetic approach toward investigation of the physiological role of specific cyclophilins. The sensitivity of yeast to CsA has been shown to depend on yeast CyPA in susceptible strains (Tropschug et al., 1989). Availability of pure yCyPA and yCyPB proteins has permitted determination of sensitivity to CsA in vitro. Characterization of the pure recombinant proteins revealed catalytic efficiency comparable to that of human cyclophilins and similar susceptibility to inhibition by CsA (Table 1). We have recently shown that both hCyPA and hCyPB (Swanson et al., 1992) can present CsA for potent inhibition of the protein serine phosphatase ability of calcineurin, a target discovered by Liu et al. (1991b). The recent cloning and overexpression of yeast calcineurin homologs (Cyert et al., 1991) will permit assessment of whether these yeast cyclophilins in combination with bound CsA inhibit yeast calcineurins and then set the stage for genetic and mutational approaches to dissect the mutual recognition of CyP, CsA, and calcineurin in yeast systems.

The question of whether cyclophilins and the FK binding protein class of PPIases (FKBPs) are essential for viability has been examined. Studies on combinations of yeast CyPA, CyPB, and yeast FKBP mutants do not reveal a startling phenotype on disruption of both types of proteins (Heitman et al., 1991; Koser et al., 1991). If these proteins are indeed functioning as protein-folding catalysts, there may be gene redundancy for one or more cell compartments, or the background folding rates in the absence of cyclophilins may suffice under most growth conditions. Additional CyP and/or FKBP genes may have to be disrupted before requirements for particular PPIases are demonstrable in yeast.

Materials and methods

Materials

The PCR kit and Ampli*Taq* DNA polymerase were purchased from Perkin-Elmer/Cetus. Sequenase was purchased from United States Biochemicals. The tetrapeptide substrate AAPF, α-chymotrypsin, and biochemicals used in the enzyme purification were purchased from Sigma Chemical Co. Biochemicals and chromatography materials used to purify the antibodies (DEAE Affi-Gel Blue and Affi-Gel-10) used in the immunochemical studies were purchased from Bio-Rad. Cyclosporin A was a generous gift from Sandoz. PCR was performed on a Perkin-Elmer/Cetus DNA Thermal Cycler. Enzyme assays were performed on a Hewlett-Packard 8452 diode array spectrophotometer, and the first order rate constants were derived as previously described (Liu et al., 1990). Fluorescence spectroscopy was performed on a Shimadzu RF-5000 spectrofluorophotometer. Isoelectric focusing was performed on a Pharmacia PhastSystem using a Bio-Rad broad range pI calibration kit. For Northern analysis, probes used for screening were labeled

by random priming with the Prime-It kit from Stratagene. Developing solutions and antibody-alkaline phosphatase conjugate for the Western blotting were obtained from Bio-Rad. Donkey antirabbit IgG ^{125}I-tagged antibody was obtained from Amersham. Nitrocellulose filters for Northern and Western blotting were obtained for Schleicher and Schuell.

The PCR primers used to amplify the yCyPA and yCyPB genes for overexpression are as follows. For the N-terminal primers for *yCyPA* and *yCyPB*, LZ#22 and LZ#38, LZ#54, respectively, the Shine/Delgarno (S/D) sequences are underlined, and the ATG start codons are in bold face. The corresponding C-terminal primers, LZ#23 and LZ#25, have the sequences complementary to the yeast genes in bold face. Primer LZ#22 (*yCyPA*), 5′-GT CGA CGA ATT CAA GGA GAT ATA CAT **ATG** TCC CAA GTC TAT TTT GA-3′; LZ#23 (*yCyPA*) 5′-CTA GGA TCC AAG CTT **AGT TAG TTA TAA GAA TTG ATT**-3′; LZ#38 (*yCyPB*, genetically truncated, minus 20 amino acids) 5′-GT CGA CGA ATT CCT GCA GAA GGA GAT ATA CAT **ATG** TCT GAT GTG GGT GAG TTG ATT GAT-3′; LZ#54 (*yCyPB*, full length) 5′-AA GCT TGT CGA CGA ATT CCT GCA GAA GGA GAT ATA CAT **ATG** AAA TTC AGT GGC TTG TG-3′; LZ#25 (*yCyPB*) 5′-CTA GGA TCC AAG CTT **ACG TTC CGG TGG TCA CAG TGC**-3′.

Methods

Construction of an overexpression vector for yCyPA and yCyPB

The *yCyPA* and *yCyPB* coding sequences were amplified using PCR with the primers, LZ#22 and LZ#23 or LZ#38 (genetically truncated) and LZ#25, and genomic yeast DNA as the template. The PCR mixture (100 μL) contained the following: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM dNTPs, 25 pmol of the corresponding *yCyPA* and *yCyPB* PCR primers, DNA template, and 2.5 units of *Taq* DNA polymerase. After an initial 5 min at 94 °C, PCR was carried out for 30 cycles; each cycle consisted of 1 min at 94 °C, 1 min at 55 °C, and 6 min at 72 °C. At the end of the 30 cycles, the sample was cooled to room temperature and incubated with Klenow enzyme for 30 min at 37 °C. Amplification resulted in a DNA fragment containing the coding sequence for yCyPA flanked on the 5′ end by an S/D with the restriction sites *Sal* I and *Eco* RI and on the 3′ end by the restriction sites *BamH* I and *Hind* III. The DNA fragment containing the coding sequence for yCyPB was flanked on the 5′ end by an S/D sequence with the restriction sites *Sal* I, *Eco* RI, and *Pst* I and on the 3′ end by the restriction sites *BamH* I and *Hind* III. The purified PCR fragments were digested with *Eco* RI and *BamH* I and subcloned into pKen. The resulting plasmids, pKLZYA and pKLZYB, were transformed into competent *E. coli* XA90 (Ausubel et al., 1987; Maniatis et al., 1989).

Protein purification

A 1-L culture of the pKLZYA-transformed *E. coli* (Luria broth, 37 °C, 100 μg/mL ampicillin) was grown to an OD_{600} of 0.6, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Bachem), and harvested 6–9 h after induction, yielding approximately 6 g/L of cells (wet weight). The cells were lysed by two passes through a French press at 1,200 psi. After centrifugation (20,000 × *g*, 25 min), nucleic acids were precipitated by protamine sulfate (0.4% final concentration). Centrifugation (20,000 × *g*, 20 min) gave a supernatant that was fractionated with ammonium sulfate. The 40–60% pellet was resuspended in 40 mL of 20 mM Tris-HCl (pH 7.8) and dialyzed overnight against 4 L of 20 mM Tris-HCl (pH 7.8). The protein was purified on a DEAE-Sepharose CL-6B (2.5 × 27 cm) eluting with 20 mM Tris-HCl (pH 7.8) (Liu et al., 1990), followed by a Sephadex G-75 (2.5 × 90 cm) column eluted with 10 mM potassium phosphate (pH 7.5)/50 mM KCl/0.02% NaN_3. This yielded 58 mg of the 17.4-kDa yCyPA protein with greater than 90% purity.

Yeast CyPB protein (genetically truncated) was purified from XA90/pKLZYB (4–6 g/L) as previously described, with the following modifications: an ammonium sulfate fractionation of 60–75% was chromatographed on a DEAE-Sepharose CL-6B (2.5 × 27 cm) column eluting with an NaCl gradient (0–750 mM NaCl, 500 mL) in 20 mM Tris HCl (pH 7.8), followed by a Sephadex G-75 (2.5 × 90 cm) column eluted with 10 mM potassium phosphate (pH 7.0)/250 mM KCl/0.02% NaN_3. This yielded 10.0 mg of the genetically truncated 20.5-kDa yCyPB protein of greater than 90% purity.

Protein characterizations

The protein concentrations were determined by Bradford assays, and proteins were analyzed on 15% denaturing SDS/polyacrylamide gels.

PPIase activity assay. The enzyme assay employed was a modification of an assay using the tetrapeptide substrate, AAPF (Fischer et al., 1984a,b). The assay was performed at 10 °C, in 35 mM HEPES (pH 8.0), 100 μM AAPF (dissolved in 66% v/v dimethyl sulfoxide), and 250 μg/mL α-chymotrypsin, with varying CyP concentrations (3.45–58 nM). The reaction mixture was preincubated at 10 °C, and the reaction was initiated by the addition of α-chymotrypsin. An absorbance reading at 390 nm was taken every 0.5 s. First order rate constants were calculated from raw data by an HP 9000-300 computer.

IC_{50} determination. The enzyme assay described above was performed using enzyme concentrations of 18 nM with the addition of CsA (dissolved in ethanol) at various concentrations in the range of 0–1,000 nM. To calculate the half-maximal inhibitory concentration (IC_{50}), percent remaining activity was plotted against the common logarithm of the CsA concentration, and these data

were fit to the following equation, using KaleidaGraph software published by Synergy Software: $y = y_{max}/(1 - [CsA]/IC_{50})$.

Determination of pI by isoelectric focusing (IEF). The pIs of both yeast cyclophilins were determined using a Pharmacia PhastSystem. Approximately 500 ng of protein was loaded onto an IEF 3-9 PhastGel, focused using the Pharmacia separation technique file #100 and stained with Coomassie blue R250. The pIs of the proteins were determined by direct comparison to standard proteins of known pI run on the gel.

Preparation of antibodies against yeast cyclophilins

YCyPA or yCyPB was purified from the XA90/pKen overexpression system as described. On day 0, specific pathogen-free New Zealand White female rabbits (Hazelton Labs), weighing 3–4 kg, were primed intramuscularly with FCA. On day 7, popliteal lymph node immunization of two rabbits was performed (200 μg yCyPA or yCyPB/rabbit in FCA). Each rabbit was boosted on day 14 with an intradermal injection of the appropriate yCyP (100 μg/rabbit) in IFA and boosted again on day 28 with a subcutaneous injection of the appropriate yCyP (50 μg/rabbit) in IFA. Rabbits were subsequently boosted monthly with yCyPA or yCyPB (50 μg in IFA) and bled 14 days after each boost.

Purification of antibodies against yeast cyclophilins

Antisera (2.5–7.5 mL) were dialyzed against 20 mM Tris-HCl/28 mM NaCl (pH 8.0) overnight at 4 °C. Column chromatography using DEAE Affi-Gel Blue (1 × 12 cm) was used to prepare a protease-free IgG fraction eluted with 20 mM Tris-HCl/28 mM NaCl (pH 8.0). Antibodies against yCyPA were further purified by selective affinity chromatography using an Affi-Gel column coupled with yCyPA (Harlow & Lane, 1988). Western immunoblotting and enzyme-linked immunosorbent assays were used to determine the specificity of the purified antibodies.

Enzyme-linked immunosorbent assay for antibody specificity

YCyPA or yCyPB (10 μg/mL) was adsorbed to the wells of Maxisorp microtiter plates (Nunc). The wells were blocked with diluting buffer (0.25% BSA, 0.05% Tween 20 in PBS, pH 7.2) and then incubated with the antibody samples (1:10 dilutions of column fractions, or serial dilutions of purified antibodies) for 0.5–1 h at 37 °C. After washing three times with wash buffer (0.05% Tween 20 in PBS), the wells were incubated with Bio-Rad goat antirabbit-horseradish peroxidase conjugate (1:1,000 in diluting buffer) for 0.5–1 h at 37 °C and washed three times with wash buffer. Peroxidase substrate solution (2 mM 2,2′-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid], 0.045% H_2O_2, 24 mM sodium citrate, 52 mM sodium phosphate, pH 5.2) was added to each well. After 30 min at room temperature, the absorbance at 405 nm was read in a V_{max} Kinetics Molecular Devices microplate reader.

Western immunoblotting

A 15% SDS-polyacrylamide gel was run of the proteins of interest, and the proteins were transferred to nitrocellulose at 150 mA in 50 mM Tris/600 mM glycine/20% methanol over 6–8 h. The membrane was treated with blocking solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% Carnation nonfat dry milk) for 2 h at room temperature. The blocking solution was removed, and the membrane was then treated with antisera from rabbits immunized with hCyPA or yCyPA (1:300 in blocking solution) overnight at 4 °C. After washing four times (1 × 1 min, 2 × 20 min, 1 × 1 min) with the blocking solution, the membrane was treated with goat antirabbit-alkaline phosphatase conjugate (1:1,500 in blocking solution, Bio-Rad 170-6518), or 20 μCi ^{125}I-tagged donkey antirabbit IgG antibody in blocking solution (Amersham), for 1 h at room temperature. The membrane was washed once for 5 min with blocking solution, 4× for 5 min with PBS (pH 7.2) and finally developed in freshly prepared developer at room temperature (10 min–4 h). Developer contained 50 mL of carbonate buffer (0.1 M $NaHCO_3$, pH 9.8, 1 mM $MgCl_2$), 0.5 mL nitroblue tetrazolium chloride solution (Bio-Rad 170-6532), and 0.5 mL 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt solution (Bio-Rad 170-6539). The development was stopped by washing in water and drying on blotting paper. If the secondary antibody was ^{125}I tagged, the filter was dried on blotting paper immediately following the PBS washes and exposed to X-ray film for 5–20 h at −70 °C with an intensifying screen.

Metabolic labeling of yeast cells with ^{35}S-methionine

For each labeled aliquot, 5×10^7 mid-log phase *S. cerevisiae* KY 320 (a) cells (OD_{600} ~1) grown in YEPD medium (Ausubel et al., 1987) at 30 °C were collected by centrifugation (4 °C, 5 min, 5,000 × *g*). The pellet was washed once in 5 mL −met medium (Ausubel et al., 1987), resuspended in 5 mL −met, and incubated for 2.5 h at 30 °C. An aliquot of 250 μCi of labeled ^{35}S-methionine (ICN) was then added, and the incubation continued for another 4–5 h. The labeled cells were collected by centrifugation (4 °C, 5 min, 5,000 × *g*), resuspended in 1 mL sterile double distilled water, transferred to a sterile Eppendorf tube, and centrifuged briefly before lysis of the cells.

Immunoprecipitation of yeast extract

Native and denatured lysis of ^{35}S-labeled yeast cells was performed according to the procedure of Kolodziej

and Young (1991). Antibody was added to each tube of native or denatured cell lysate in the following dilutions: 1:50, 1:100, 1:250, 1:500. One microliter of the prebleed was added to an additional sample as a negative control. Protein A-Sepharose beads (25 μl of a 50% solution) were added to each tube and the samples incubated at 4 °C while rotating end over end for 4 h. After centrifugation, the supernatant was removed and frozen, and the Protein A-Sepharose beads were washed three times with the lysis buffer (250 μL). Laemmli loading buffer (50 μL) was added to each sample, and the samples were subjected to 15% SDS-PAGE. After drying, the gel was exposed to X-ray film for 12–24 h at room temperature.

Preparation of yeast cell-free extract

Aliquots (1.5 mL) of cultures of *S. cerevisiae* KY 320 (a) cells grown at 30 °C in YEPD medium to mid-log phase (OD_{600} = 1–2) were harvested by microcentrifugation (15 s, 5K) and the cell pellets washed in 200 μL TE each. The washed pellets were then resuspended in 200 μL 2× Laemmli loading buffer each. Acid-washed glass beads (200 μL, 0.45–0.6 mm, Sigma) were added to each tube, and the tubes were vortexed at the highest speed for 2 min. The extracts were then immediately boiled for 3 min, frozen in liquid N_2, and stored at −20 °C. Extracts were similarly prepared of four strains of *S. cerevisiae*, the generous gift of Miles Brennan of the National Institutes of Mental Health: MB 11 (*yCyPA*$^+$, *yCyPB*$^+$), MB 12 (*yCyPA*$^-$, *yCyPB*$^+$), ED 19 (*yCyPA*$^+$, *yCyPB*$^-$), and ED 21 (*yCyPA*$^-$, *yCyPB*$^-$). Cell-free extracts were then directly subjected to 15% SDS-PAGE preparatory to Western analysis.

Preparation of yeast culture broth for Western analysis

Saccharomyces cerevisiae KY 320 (a) was grown in 1 L of YEPD medium to mid-log phase (OD_{600} = 1.7) at 30 °C. A 1.5-mL aliquot of the culture was removed, and the cell pellet extract was prepared as above. The remainder of the culture was centrifuged (2,500 × *g*, 20 min, 4 °C) to remove the cells. The pH of the pooled supernatant was adjusted to 8.0 with 10 N NaOH. DEAE-Sepharose CL-6B (250 mL, Sigma), equilibrated with 20 mM Tris-HCl (pH 8.0), was added and the culture broth stirred slowly overnight at 4 °C. The DEAE-Sepharose was then filtered through a Büchner funnel and washed with 20 mM Tris-HCl (pH 8.0). The adsorbent pad was then stirred and filtered with successive 250-mL volumes of 250, 500, 750 mM, and 1 M NaCl in buffer. The 500 mM and 750 mM NaCl filtrates were concentrated to 8 mL each, precipitated with 8% TCA, and centrifuged (8,000 × *g*, 10 min, 4 °C). Each pellet was dissolved by boiling in 1 mL of Laemmli SDS buffer, basified with 4 N NaOH, and subjected to 15% SDS-PAGE prior to Western analysis.

Northern analysis

Two cultures of *S. cerevisiae* KY 320 (a) cells were grown in YEPD medium to mid-log phase (OD_{600} = 1–2) at 30 °C. One aliquot was then heat-shocked at 37 °C for 5 min. Total RNA was isolated by glass bead disruption in 0.5 M NaCl/200 mM Tris-HCl (pH 7.5)/10 mM EDTA, followed by phenol:$CHCl_3$:isoamyl alcohol (25:24:1) extraction (Ausubel et al., 1987). As controls, samples of total RNA from MB 11, MB 12, ED 19, and ED 21 were isolated in the same manner. Each RNA sample (40 μg) was fractionated on a 1.2% agarose gel containing 20 mM 3-(*N*-morpholino)propanesulfonic acid and 0.49 M formaldehyde, transferred overnight to a polyvinylidene difluoride membrane, baked for 2 h at 80 °C, and prehybridized for 12–24 h at 45 °C in a prehybridization buffer containing 25 mM potassium phosphate buffer (pH 7.4), 5× standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhart's solution, 50 μg/mL salmon sperm DNA, and 50% formamide. The membrane was hybridized in hybridization buffer for 24 h at 45 °C with double-stranded DNA probes labeled with [α-^{32}P]dCTP (3,000 Ci/mmol for *yCyPA* probes, 6,000 Ci/mmol for *yCyPB*; NEN) by random priming on PCR-amplified fragments of the cloned genes, which were derived from cDNA (*yCyPA*) or genomic DNA (*yCyPB*). The hybridization buffer was the prehybridization buffer with the addition of 10% dextran sulfate. The membrane was washed at room temperature in 1× SSC/1% SDS for 30 min, then four times at 45 °C for 15 min in 0.2× SSC/0.1% SDS. The washed membrane was blotted dry on filter paper and exposed to X-ray film for 20–50 h at −70 °C with an intensifying screen.

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