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Human cyclophilin B: A second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence

(cyclosporin A/proline isomerase/rotamase/T-cell activation)

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ABSTRACT **We report the cloning and characterization of a cDNA encoding a second human cyclosporin A-binding protein (hCyPB). Homology analyses reveal that hCyPB is a member of the cyclophilin B (CyPB) family, which includes yeast CyPB, *Drosophila* nina A, and rat cyclophilin-like protein. This family is distinguished from the cyclophilin A (CyPA) family by the presence of endoplasmic reticulum (ER)-directed signal sequences. hCyPB has a hydrophobic leader sequence not found in hCyPA, and its first 25 amino acids are removed upon expression in *Escherichia coli*. Moreover, we show that hCyPB is a peptidyl-prolyl *cis–trans* isomerase which can be inhibited by cyclosporin A. These observations suggest that other members of the CyPB family will have similar enzymatic properties. Sequence comparisons of the CyPB proteins show a central, 165-amino acid peptidyl-prolyl isomerase and cyclosporin A-binding domain, flanked by variable N-terminal and C-terminal domains. These two variable regions may impart compartmental specificity and regulation to this family of cyclophilin proteins containing the conserved core domain. Northern blot analyses show that hCyPB mRNA is expressed in the Jurkat T-cell line, consistent with its possible target role in cyclosporin A-mediated immunosuppression.**

The cyclophilins (CyPs) are a conserved class of proteins that bind the immunosuppressive drug cyclosporin A (CsA) with high affinity (K_d = 5–200 nM) (1, 2). CsA blocks helper T-cell activation at a step between T-cell receptor stimulation and the transcriptional activation of cytokine genes (encoding interleukin 2, interleukin 3, granulocyte–macrophage colony-stimulating factor, γ-interferon) (3, 4). Because the affinities of CsA derivatives for CyP correlate with their immunosuppressive activities, CyP has been implicated in the helper T-cell signal transduction pathway (2, 5). CyPs from many species possess peptidyl-prolyl *cis–trans* isomerase (PPIase) activity which is blocked by CsA and therefore may be relevant in CsA-mediated immunosuppression (6, 7).

Definitive support for the idea that CyP plays a critical role in T-cell signal transduction has not been presented. Surprisingly, CyPs are found in all cell types examined, at high concentrations not likely to be saturated by therapeutic levels of CsA (1, 2, 8). Moreover, CsA affinity columns bind an array of proteins in addition to the major CyP species (2, 9). Therefore, the formal possibility exists that the therapeutically relevant target is a less abundant protein with a higher affinity for CsA. To elucidate the mechanism by which CsA interferes with T-cell activation and to understand the role of CyPs in the cell, intense efforts have focused on the interactions of CsA with human CyP and its homologues in mammalian, fungal, and prokaryotic species. To expand this effort to other potential cellular targets of CsA, human cDNA libraries were probed with the only known human CyP cDNA under reduced stringencies, and a second CyP gene, encoding human CyP B (hCyPB), was discovered.‡ The protein is 64% identical to human CyP A (hCyPA) and is distinguished from it by a signal sequence that probably directs it to the endoplasmic reticulum (ER). hCyPB has even stronger similarity to yeast CyPB (yCyPB) (10), which also has an ER-directed signal sequence. The signal sequence is removed from hCyPB upon expression in *Escherichia coli*, and the processed protein possesses PPIase activity that is inhibited by CsA.

MATERIALS AND METHODS

Materials. The polymerase chain reaction (PCR) kit and *Thermus aquaticus* (*Taq*) DNA polymerase were purchased from Perkin–Elmer/Cetus. Sequenase was purchased from United States Biochemical. The tetrapeptide substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, α-chymotrypsin, and biochemicals used in the enzyme purification were purchased from Sigma. The Jurkat cDNA library was obtained from Clontech. The endothelial cDNA was kindly provided by D. Littman (University of California, San Francisco). CsA was a generous gift from Sandoz. Nylon membranes (Biotrans) used in the Northern blot analyses were obtained from ICN.

Enzyme assays were performed on a Hewlett–Packard 8452 diode array spectrophotometer, and the first-order rate constants were derived as previously described (11). The Northern blot analyses were quantitated by using a Betascope detector (Betagen, Waltham, MA).

The PCR primers used to amplify the human CyPB gene for overexpression are as follows. For the N-terminal primer (LZ#33), the Shine–Dalgarno sequences are underlined, and the ATG start codons are in boldface. The corresponding C-terminal primer (LZ#34) has the complementary sequence to the hCyPB gene in boldface. Primer LZ#33, 5′-GT CGA CGA ATT CCT GCA GAA GGA GAT ATA CAT **ATG** AAG GTG CTC CTT GCC GCC GCC-3′; LZ#34 5′-GAG CTC CTA GGA TCC AAG CTT **CTA CTC CTT GGC GAT GGC** AAA-3′.

Library Screening and Northern Analysis. λGT10 cDNA libraries made from endothelial cells and Jurkat cells were screened by hybridization with the human CyP cDNA (11) labeled by random priming (12). Hybridization was performed at 40°C in 50% (vol/vol) formamide/5× SSC/5× Denhardt's solution/0.1% SDS containing salmon sperm DNA at 150 μg/ml (ref. 13, sect. 1.90; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7, and 1× Denhardt's

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Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; h-, y-, and r-, human, yeast, and rat; PPIase, peptidyl-proline *cis–trans* isomerase; rCyLP, rat CyP-like protein; ER, endoplasmic reticulum.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M60857).

solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). Filters were washed extensively with 0.3× SSC/0.1% SDS at 50°C, and radioactivity was detected by using a 14-hr exposure to Kodak XAR-5 film with an intensifying screen. Sequence determination was performed, after cloning the cDNA in the *Eco*RI site of the Bluescript KS vector, using Sequenase-directed chain termination methods according to the supplier (United States Biochemical).

Total RNA was prepared from HeLa and Jurkat cells by the lithium chloride method (14). The RNA (20 μg) was fractionated on a 1% agarose gel containing 20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) and 2.2 M formaldehyde, transferred overnight to a nylon membrane, and probed with the hCyPA and hCyPB cDNAs in a hybridization buffer containing 7% SDS, 1% bovine serum albumin, and 0.5 M sodium phosphate buffer (pH 7.2) at 65°C for 24 hr (15). The membranes were washed twice for 20 min in 2× SSC/0.2% SDS at room temperature, followed by two washes in the same solution at 65°C. Signals were quantitated by using a Betascope detector (Betagen).

Construction of an Overexpression Vector for hCyPB. The hCyPB coding sequence was amplified by PCR using the

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 1  CGGGAACGCAACATGAAGGTGCTCCTTGCCGCCGCCCTCATCGCGGGGTCCGTCTTCTTC
                M  K  V  L  L  A  A  A  L  I  A  G  S  V  F  F

61  CTGCTGCTGCCGGGACCTTCTGCGGCCGATGAGAAGAAGAAGGGGCCCAAAGTCACCGTC
     L  L  L  P  G  P  S  A  A  D  E  K  K  K  G  P  K  V  T  V

121 AAGGTGTATTTTGACCTACGAATTGGAGATGAAGATGTAGGCCGGGTGATCTTTGGTCTC
     K  V  Y  F  D  L  R  I  G  D  E  D  V  G  R  V  I  F  G  L

181 TTCGGAAAGACTGTTCCAAAAACAGTGGATAATTTTGTGGCCTTAGCTACAGGAGAGAAA
     F  G  K  T  V  P  K  T  V  D  N  F  V  A  L  A  T  G  E  K

241 GGATTTGGCTACAAAAACAGCAAATTCCATCGTGTAATCAAGGACTTCATGATCCAGGGC
     G  F  G  Y  K  N  S  K  F  H  R  V  I  K  D  F  M  I  Q  G

301 GGAGACTTCACCAGGGGAGATGGCACAGGAGGAAAGAGCATCTACGGTGAGCGCTTCCCC
     G  D  F  T  R  G  D  G  T  G  G  K  S  I  Y  G  E  R  F  P

361 GATGAGAACTTCAAACTGAAGCACTACGGGCCTGGCTGGGTCAGCATGGCCAACGCAGGC
     D  E  N  F  K  L  K  H  Y  G  P  G  W  V  S  M  A  N  A  G

421 AAAGACACCAACGGCTCCCAGTTCTTCATCACGACAGTCAAGACAGCCTGGCTAGATGGC
     K  D  T  N  G  S  Q  F  F  I  T  T  V  K  T  A  W  L  D  G

481 AAGCATGTGGTGTTTGGCAAAGTTCTAGAGGGCATGGAGGTGGTGCGGAAGGTGGAGAGC
     K  H  V  V  F  G  K  V  L  E  G  M  E  V  V  R  K  V  E  S

541 ACCAAGACAGACAGCCGGGATAAACCCCTGAAGGATGTGATCATCGCAGACTGCGGCAAG
     T  K  T  D  S  R  D  K  P  L  K  D  V  I  I  A  D  C  G  K

601 ATCGAGGTGGAGAAGCCCTTTGCCATCGCCAAGGAGTAGGGCACAGGGACATCTTTCTTT
     I  E  V  E  K  P  F  A  I  A  K  E  @

661 GAGTGACCGTCTGTGCAGGCCCTGTAGTCCGCCACAGGGCTCTGAGCTGCACTGGCCCCG

721 GTGCTGGCATCTGGTGGAGCGGACCCACTCCCCTCACATTCCACAGGCCCATGGACTCAC

781 TTTTGTAACAAACTCCTACCAACACTGACCAATAAAAAAAAATGTGGGTTTTTTTTTTT

841 TTAATAAAAAA
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human CyPA: M.............................VNPTVFFDIAVDGEPLGRVSFELFADK
human CyPB: -kvllaaaliagsvffllpgpsaadekkkgpk-TVK-Y--LRIGD-DV---I-G--GKT
yeast CyPB: -kfsglwcwlllflsvnviasdvgelidqddevITQK-----EHGE-KV--IVIG-YGKV
  rat CyLP: -kvlfaaalivgsvvfllpgpsvandkkkgpk-TVK-Y--FQIG-RTCRTSDLWTLWKD

human CyPA: VPKTAENFRALS.TGEKGFGYKGSCFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDEN
human CyPB: ----VD--V--A.---------N-K---V-KD--I-------GD----------R-P---
yeast CyPB: C----K--YK--t-TNSKK-FI--T---V--N--V------DGT-V-------DT-P---
  rat CyLP: CS--VD--V--A.---------N-K--HM-KD--I-------GD----------R-P---

human CyPA: FILKHTGPGILSMANAGPNTNGSQFFI.CTAKTEWLDGKHVVFGKVKEGMNIVEAME.RF
human CyPB: -K---Y---WV------KD--------.T-V--A-----------L---EV-RKV-sTK
yeast CyPB: -T---DRK-R-----R-KD--------tT-EEAS----------Q-VD--DV-NYIQ.HV
  rat CyLP: -K---Y---TV------KD--------.T-V--S-----------L---DV-RKV-nTK

human CyPA: G.SRNGKTSKKITIADCGQLE
human CyPB: T.DSRD-PL-DVI-----KI-VEKPFAIAKE
yeast CyPB: SrDA-D-PLEAVK--K--EWTPELSS
  rat CyLP: T.DSRD-PL-DVI-V---KI-VEKPFAIAKELREPRGPHPSKQLSVRVLSIPHR
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FIG. 1. (*Upper*) Nucleotide and deduced amino acid sequence of hCyPB. (*Lower*) Amino acid sequence alignment showing homologies between human CyPA (165 amino acid residues), human CyPB (208 residues), yeast CyPB (206 residues), and rat CyP-like protein (rCyLP) (231 residues). Lowercase letters indicate insertions relative to hCyPA at the N terminus, dots indicate deletions, and hyphens indicate identities.

primers LZ#33 and LZ#34 and the isolated cDNA fragment 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% gelatin, 200 μM dNTPs, 25 pmol of LZ#33 and LZ#34, DNA template, and 2.5 units of *Taq* DNA polymerase. The 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 the Klenow fragment of DNA polymerase for 30 min at 37°C. Amplification resulted in a DNA fragment containing the coding sequence for hCyPB flanked on the 5′ end by a Shine–Dalgarno sequence with the restriction sites for *Sal* I, *Eco*RI, and *Pst* I and on the 3′ end by the restriction sites for *Sac* I, *Bam*HI, and *Hin*dIII. The purified PCR fragment was digested with *Eco*RI and *Bam*HI and subcloned in pKen, and the resulting plasmid, $pKLZHB_5$, was used to transform competent *E. coli* XA90 (ref. 13, sect. 1.82; ref. 16, sect. 1.81).

Protein Purification. A 1-liter culture of the $pKLZHB_5$-transformed *E. coli* (LB medium, 37°C, with ampicillin at 100 μg/ml) was grown to an OD_{660} of 0.6, induced with 1 mM isopropyl β-D-thiogalactopyranoside, and harvested 9 hr later, yielding approximately 5 g/liter of cells (wet weight). The cells were lysed by two passes through a French press at 16,000 psi (110 MPa). After centrifugation (20,000 × *g*, 25 min), the protein was purified (11), yielding 1 mg of a 21-kDa protein of greater than 90% purity.

Protein Characterizations. The protein concentrations were determined by Bradford assays (17), and the proteins were analyzed on denaturing SDS/15% polyacrylamide gels.

CyP activity assay. The enzyme assay employed was a modification of an assay using the tetrapeptide substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (AAPF) (18, 19). The assay was performed at 10°C, in 35 mM Hepes buffer (pH 8.0), 100 μM AAPF [dissolved in 66% (vol/vol) dimethyl sulfoxide], and α-chymotrypsin at 250 μg/ml, while CyP concentration was varied (3.45–58 nM). The reaction mixture was incubated 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 sec.

IC_{50} determination. The enzyme assay described above was performed with the addition of various final concentrations of CsA (dissolved in ethanol) in the range of 0–1000 nM.

RESULTS

Isolation and Sequencing an hCyPB cDNA. To search for related human CyP cDNAs, the hCyPA cDNA (11) was used to screen human endothelial and Jurkat T-cell cDNA libraries. As described in *Materials and Methods*, several candidate clones yielding lower hybridization signals than hCyPA were analyzed by restriction mapping. All five yielded identical maps which were distinct from the hCyPA map. The DNA and deduced amino acid sequence of the longest cDNA are presented in Fig. 1 *Upper*. This cDNA has an open reading frame encoding 208 amino acids and shows a high degree of identity with yCyPB (Fig. 1 *Lower*). Therefore, this protein will be referred to as hCyPB.

A sequence alignment and comparison of hCyPB with hCyPA, yCyPB, and rCyLP (20) revealed a set of domains which have various degrees of conservation. These domains are schematically presented in Fig. 2, and they have been labeled domains I through VI. From Figs. 1 *Upper* and 2, it is clear that hCyPB is more structurally similar to yCyPB and rCyLP than it is to hCyPA. The 33-amino acid domain I in hCyPB, yCyPB, and rCyLP contains characteristic ER-directed signal sequences (21, 22) and is absent from hCyPA. Domain II has 61 amino acids and at least 46% identity between the two families of CyPs. Domain III has 77 amino acids and represents the most conserved region of the larger core domain and contains the conserved tryptophan thought to interact with CsA (refs. 23 and 24; J. Liu, C.-M. Chen, and C. T. Walsh, unpublished results). Domain II and domain III of hCyPB have maintained a higher degree of conservation to hCyPA than they have to nina A and yeast CyPB, both nominally CyPB proteins. Domain IV, which has 27 amino acids, is the least conserved region of the core domain shared by both families of CyPs. Domain V of hCyPB has 10 amino acids not found in the CyPA family of proteins. Domain V of yCyPB has only 5 amino acids with no obvious homology to

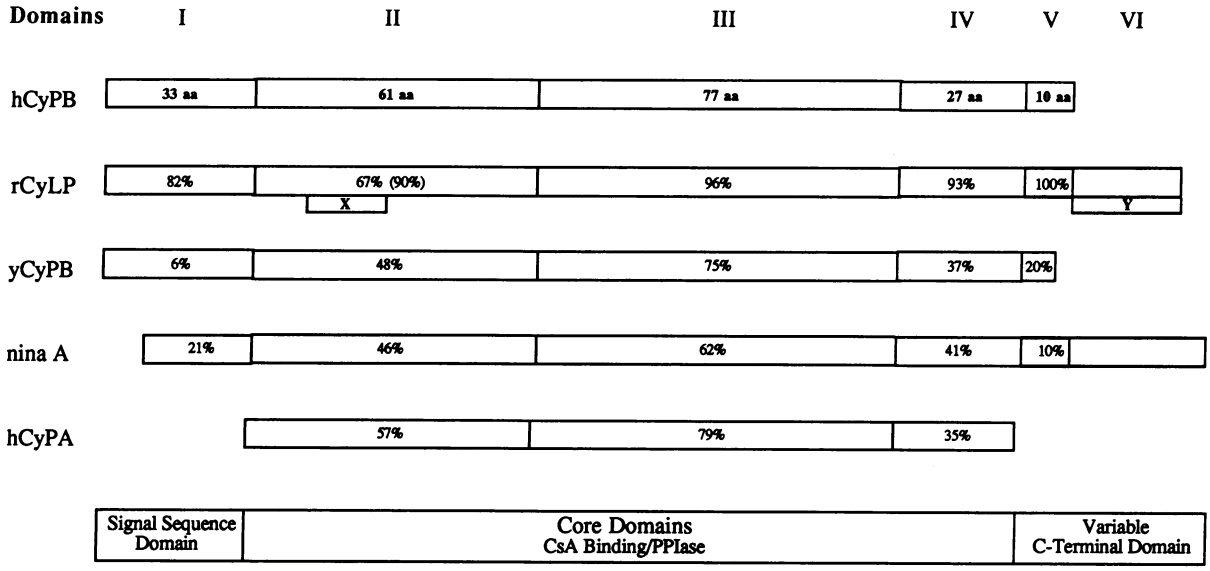

FIG. 2. Schematic comparison of hCyPB to various CyP homologues. Six domains are suggested and further described in the text. For each domain, the percentage of amino acid identity between human CyPB and a particular homologue is displayed. The CyPB family is distinguished from the CyPA family by additional domains found at both the N and C termini of a central core which harbors CsA binding and PPIase activity. The bars under rCyLP indicate regions that are strikingly different from the highly homologous hCyPB sequence. Although these areas may indicate regions of functional variability, they could instead embody frameshifting errors. An alternate reading frame at region X yields a sequence with only 2 mismatches instead of 15 over 17 residues. This change in sequence increases the amino acid identity from 67% to 90%. Region Y disappears altogether if a single base insertion that creates a stop codon is postulated at the beginning of region Y.

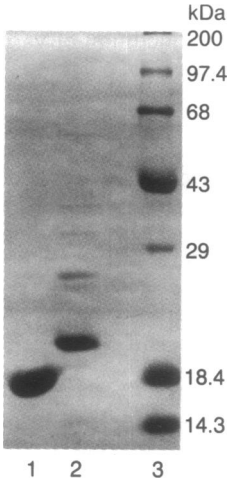

FIG. 3. SDS/PAGE analysis of hCyPA and hCyPB purified from *E. coli*. Lane 1 has 20 μg of hCyPA; lane 2 has 20 μg of hCyPB; and lane 3 has molecular mass standards.

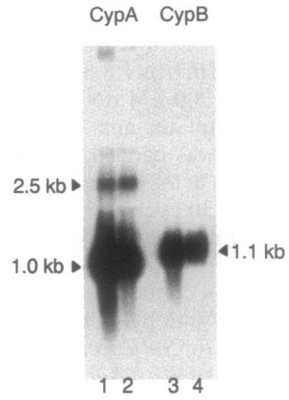

FIG. 4. Northern blot analyses of hCyPA and hCyPB mRNA levels in HeLa and Jurkat cells. Lanes 1 and 3 represent HeLa cell levels of CyPA and CyPB mRNA, respectively. Lanes 2 and 4 represent Jurkat cell mRNA levels of CyPA and CyPB, respectively. Equivalent amounts of total RNA were loaded in each lane. The identity of the 2.5-kilobase (kb) species seen in lanes 1 and 2 is not known, but it may be another mRNA with a high degree of homology to hCyPA mRNA.

domain V in hCyPB. hCyPB and rCyLP are identical in domain V, but rCyLP apparently continues for an additional 23 amino acids represented by domain VI (Fig. 2).

Expression of hCyPB in *E. coli*: Purification and Characterization. Following the general strategy for the purification of hCyPA (11), hCyPB was expressed and purified from *E. coli*. As shown in Fig. 3, recombinant hCyPB expressed in *E. coli* yields a protein of about 21 kDa, approximately 3–4 kDa larger than hCyPA. The N-terminal sequence analysis of the *E. coli*-expressed hCyPB protein (DEKKKGPKVTVKVYF, which corresponds to residues 26–40 in hCyPB) (Fig. 1 *Upper*) indicated that the first 25 residues had been removed in *E. coli*. It has not been assessed whether hCyPB is secreted into the *E. coli* periplasm as is the *E. coli* CyP (25).

Pure hCyPB is an active PPIase as determined in the standard tetrapeptide chromogenic PPIase assay. As depicted in Table 1, hCyPB shows a k_{cat}/K_m of 6.3×10^6 $M^{-1} \cdot s^{-1}$. When recombinant hCyPA was assayed in parallel, its catalytic efficiency was found to be 2.5-fold less than that of hCyPB. The hCyPB PPIase activity at an enzyme concentration of 6.9 nM was found to be inhibited by CsA with an IC_{50} of 84 ± 13 nM. hCyPA at the same concentration was approximately 1/3rd as sensitive to CsA inhibition.

mRNA Detection in Human Cell Lines. Northern blot analyses were performed on RNA from HeLa and Jurkat cells, using the hCyPA or hCyPB cDNAs as probes. As shown in Fig. 4, both forms of hCyP mRNA are detected in the two cell types. A 4- to 6-fold higher signal intensity was detected in the HeLa compared to the Jurkat cells for both hCyPA and hCyPB probes. hCyPA mRNA is generally about 5-fold more prevalent than hCyPB mRNA, as estimated on the basis of Northern signal intensity and their relative abundance in the two cDNA libraries.

DISCUSSION

We report the characterization of a cDNA encoding a CsA-binding protein, hCyPB, that has strong resemblance to the major cellular cyclophilin, hCyPA. In addition to this structural similarity, a functional analysis of the *E. coli*-expressed hCyPB demonstrated that it has PPIase activity which is sensitive to CsA. A distinguishing feature of hCyPB is the presence of an N-terminal signal sequence that is removed upon expression in *E. coli*. This signal sequence has characteristics common to those found in ER-directed proteins, which suggests that hCyPB will be found within the ER or in distal compartments along the secretory pathway (21, 22).

Table 1. Kinetic and IC_{50} values for the human CyPs

CyP	k_{cat}/K_m, $M^{-1} \cdot s^{-1}$	CsA IC_{50}, nM
hCyPA	1.6×10^7	25 ± 5
hCyPB	6.3×10^6	84 ± 13

The existence of a second CyP in humans is paralleled in the yeast *Saccharomyces cerevisiae*, for which two proteins with CyP homology have been reported. The first, yCyPA (26), is homologous to hCyPA (27). The second, yCyPB (10), is unusual in that it contains a signal sequence and had no previously recognized human counterpart. Comparison of the human and yeast CyPs clearly shows that hCyPB is more similar to yCyPB than to yCyPA and suggests that human and yeast CyPB were derived from a common ancestral gene.

Recently, both CyP and FKBP, an unrelated protein that binds the immunosuppressant FK506, were shown to have PPIase activity (28, 29). These observations have focused attention on PPIase activity in helper T-cell signaling pathways. hCyPA and hCyPB were compared for catalytic efficiency as PPIases and for the relative sensitivity of this activity to CsA. While both are quite active catalysts, the 2.5-fold higher activity of hCyPA, coupled with a 3-fold higher abundance of mRNA, might suggest that hCyPA is responsible for 75–80% of the total PPIase activity associated with the two CyPs in T cells. Further, hCyPA is reproducibly 3-fold more sensitive to CsA inhibition than hCyPB (25 nM vs. 84 nM IC_{50} values). However, it is not clear whether the tetrapeptide substrate used in the PPIase assay is an accurate measure of catalytic activity towards specific proteins in the cell.

A clear role for CyPs in the T-cell activation pathway blocked by CsA has not been defined. At present, the best paradigm is the CyP-like *ninaA*-encoded protein of *Drosophila*, whose mutation results in photoreceptor cell dysfunction (30, 31). It has been suggested that this phenotype is due to improper folding of rhodopsin molecules in the photoreceptor cells. The N-terminal signal sequence and the C-terminal hydrophobic domain of nina A indicate its target to be the ER, where the rhodopsin molecules would first require proper folding. While major aspects of the nina A model need clarification, it highlights the potential of tissue-specific CyPs affecting specific pathways. Although both hCyPA and hCyPB have been found in all cell types examined, cellular compartmentalization is likely to functionally distinguish the two. While hCyPA is reported to be cytosolic, the signal

sequence of hCyPB would dictate that it functions in the ER or more distal portions of the secretory pathway. Because hCyPB lacks a Lys-Asp-Glu-Leu ER-retention signal found in most ER-resident proteins (32–35), assignment of definitive subcellular localization and secretory potential will require immunocytochemical studies. The possible secretion of hCyPB may have important clinical ramifications for patients undergoing CsA therapy.

The data presented here suggest that CyP diversity can arise by additions of N-terminal and C-terminal extensions flanking the central CyP core domain. This central core domain is likely to include both the PPIase catalytic site and the CsA-binding site. While both hCyPA and hCyPB are expressed in T cells and are potential mediators of the immunosuppressive effects of CsA, additional T-cell-specific CyP genes or transcriptional variants of CyPA and CyPB may play critical roles in T-cell activation.

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