Human Cyclophilin C: Primary Structure, Tissue Distribution, and Determination of Binding Specificity for Cyclosporins

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ABSTRACT: A complementary DNA (cDNA) for human cyclophilin C (Cyp-C) was isolated from a human kidney cDNA library. Northern blot experiments with several human tissues and cell lines revealed that Cyp-C is less abundant than Cyp-A. The amount of Cyp-C mRNA was 10-fold lower than that of Cyp-A in kidney. Expression of human Cyp-C in the kidney is not significantly elevated compared to pancreas, skeletal muscle, heart, lung, and liver. This argues against a previously postulated specific role for Cyp-C in the nephrotoxic effects of CsA in humans, based on the studies of its relative abundance in murine kidney. It is present in extremely low concentrations in brain and in the Jurkat T cell line. The binding of recombinant human Cyp-A, -B, and -C to cyclosporin A (CsA) was studied by immunochemical methods. The relative affinity of Cyp-C for CsA is lower by a factor of 2 than that of Cyp-A, which itself is 10-fold lower than that of Cyp-B. Cross-reactivity studies with a series of Cs derivatives showed that Cyp-C binds CsA with a fine specificity similar to that of Cyp-A and Cyp-B. Cs amino acid residues 1, 2, 10, and 11 seemed essential for the interaction with all three Cyp subtypes. However, Cyp-C tolerates a greater variety of structures on Cs at position 2 than Cyp-A does, suggesting that this residue of CsA might not be in tight contact with Cyp-C. This was confirmed by modeling of human Cyp-C on the structure of the complex formed by Cyp-A and CsA. The knowledge of the fine specificity of human Cyps for CsA and of their expression levels may provide better insights into how CsA acts on its different target proteins *in vivo*.

Cyclosporin A (CsA),[1] an inhibitor of T cell activation, is extensively used to prevent graft rejection after organ transplantation [for review, see Borel (1989)]. CsA binds to a cytosolic protein, cyclophilin A (Cyp-A; Handschumacher et al., 1984; Haendler et al., 1987) and inhibits its peptidyl–prolyl *cis–trans* isomerase activity (Fischer et al., 1989; Takahashi et al., 1989). In the presence of CsA, cyclophilins have been shown to interact with calcineurin, a serine/threonine phosphatase dependent on Ca^{2+} and calmodulin (Liu et al., 1991; Friedman & Weissman, 1991; Li et al., 1993). According to the current model of immunosuppression, this complex formation between Cyp-A and calcineurin results in the inactivation of calcineurin and its subsequent failure to dephosphorylate NF-AT, a transcription factor present in activated T cells. Dephosphorylation is thought to be essential for the translocation of NF-AT to the nucleus, the site at which it participates in the transcription of the interleukin-2 gene (Schreiber, 1992; Liu et al., 1992; Fruman et al., 1992).

Since the discovery of Cyp-A, several homologous CsA binding proteins, collectively termed cyclophilins (Cyps), have been isolated from various organisms and tissues [for review, see Heitman et al. (1992)]. Cyps and the unrelated family of immunophilins, FK-506 binding proteins (FKBPs), have emerged as a group of compartmentalized cellular proteins involved in protein folding. A secretory form of cyclophilin, Cyp-B, has been reported (Price et al., 1991; Spik et al., 1991; Hasel et al., 1991). It is localized in the endoplasmic reticulum, possibly in calciosomes (Arber et al., 1992). Another form of human cyclophilin termed Cyp-3 or Cyp-D has been cloned by cross-hybridization with Cyp-A (Bergsma et al., 1991), and its homologs were isolated from rat and bovine mitochondria (Connern & Halestrap, 1992; Inoue et al., 1993). A second secretory cyclophilin, Cyp-C, was cloned from a murine bone marrow-derived stromal cell line (Friedman & Weissman, 1991). Cyp-C was shown to associate with calcineurin *in vitro*, in the presence of CsA, like Cyp-A and Cyp-B (Friedman & Weissman, 1991; Swanson et al., 1992). Cyp-C binds specifically to a cellular glycoprotein of 77 kDa termed Cyp-C-associated protein (CyCAP), isolated from the same murine cell line. The interaction of Cyp-C to CyCAP is inhibited by CsA, and Cyp-A and Cyp-B do not bind to CyCAP (Friedman et al., 1993). Murine Cyp-C was found to be expressed in a restricted subset of tissues, with relatively high concentrations in kidney (Friedman & Weissman, 1991; Bram et al., 1993), and was, therefore, suggested to be a relevant target for the nephrotoxic effects of CsA. The physiological roles of Cyp-C and CyCAP, however, remain to be elucidated.

Here, we report the cloning and expression of the human Cyp-C protein. In a comparative distribution study, we have analyzed the mRNA levels of Cyp-C and Cyp-A in a variety of human tissues. Furthermore, the availability of recombinant human cyclophilins A, B, and C enabled us to investigate their affinity and fine specificity for CsA. Since CsA is widely used in organ transplantation, it is important to evaluate its mechanism of action in different tissues in humans. Identifying the target proteins in humans and studying their interaction with CsA are essential parts of this understanding.

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[1] Abbreviations: Abu, α-aminobutyric acid; BSA, bovine serum albumin; cDNA, complementary DNA; Cs, cyclosporin; Cyp, cyclophilin; CyCAP, Cyp-C-associated protein; IPTG, isopropyl thiogalactoside; MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline; Nva, norvaline; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Sar, sarcosine; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

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EXPERIMENTAL PROCEDURES

Materials. Mouse kidney cDNA, human kidney total RNA, a human multiple tissue Northern blot, and a λgt10 human kidney cDNA library were obtained from Clontech, Palo Alto, CA. All Cs derivatives were from Sandoz Pharma Ltd., Basel.

cDNA Cloning and Sequencing. Cloning experiments were carried out according to Sambrook et al. (1989). A fragment of murine Cyp-C cDNA representing nucleotide residues 51–239 of the published sequence (Friedman & Weissman, 1991) was synthesized by polymerase chain reaction (PCR) using 1 ng of murine kidney cDNA as a template. The gel-purified fragment was labeled with ^{32}P by random priming. A total of 6 × 100 000 plaque-forming units of a λgt10 cDNA library from human kidney were screened by hybridization for 16 h at 55 °C in a solution containing 5 × standard saline citrate (SSC), 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), and 0.5% blocking reagent (Boehringer Mannheim). Stringent washing of filters (Hybond N, Amersham) was carried out at 55 °C in 0.2 × SSC for 40 min. Positive clones were purified by rescreening. cDNA inserts were excised with *Eco*RI, subcloned into pUC19, and sequenced by the dideoxy chain termination method (Sanger et al., 1977).

RNA Preparation and Northern Blot Analysis. Total RNA was isolated from HEK-293 and Jurkat cells using RNAzol (Cinna/Biotecx, Houston, TX) according to the manufacturer's instructions. Duplicates of 5 or 10 μg of RNA per lane were separated on a 1% agarose–formaldehyde gel and transferred to Hybond-N filters (Amersham). Full-length Cyp-C (883 bp) and Cyp-A (723 bp) cDNA was labeled with ^{32}P (Random primed DNA labeling kit, Boehringer Mannheim). The filter was divided to separate duplicates and hybridized at 42 °C in 5 × standard saline phosphate–EDTA (SSPE), 2% SDS, 10 × Denhardt's solution, 50% formamide, and 0.1 mg/mL salmon sperm DNA. Each labeled probe was added at a final concentration of 1 × 10^6 counts/mL. Stringent washing was done at 50 °C in 0.1 × SSC and 0.1% SDS. Northern blots were analyzed by PhosphorImager, and signals were quantified using MD ImageQuant version 3.2 (Molecular Dynamics). A filter containing 2 μg of immobilized poly(A)–RNA of different tissues was hybridized with a ^{32}P-labeled fragment corresponding to the first 176 residues of the 5′-end of the Cyp-C cDNA (Figure 1) excised by *Sma*I. Following signal quantification, the filter was then reprobed with a full-length Cyp-A probe.

Expression of Recombinant Cyp-C in Escherichia coli. A partial cDNA, coding for a Cyp-C polypeptide starting with the alanine residue at position 19 fused to an initial methionine, was synthesized by PCR using the oligonucleotide primer 5′-GCCCCGGCCATGGCACTTGTGTTTTCTTCGGGG-3′ and a primer hybridizing to pUC19. The resulting fragment was digested first with *Eco*RI, treated with Klenow polymerase to generate blunt ends, and subsequently cut with *Nco*I. The final fragment was cloned into the expression vector pT7-NH, a modified pET3c plasmid containing the T7 promoter (Studier et al., 1990). In pT7-NH, the *Nde*I site had been replaced by *Nco*I, and a *Hin*dIII site had been introduced downstream. The recipient vector fragment was prepared by sequential treatment of pT7-NH with *Hin*dIII, Klenow enzyme in presence of all four dNTPs, and *Nco*I. Ligation products were transformed into *E. coli* strain BL21(DE3). For induction, an overnight culture was diluted 1:6 and allowed to grow for 1 h in a culture shaker at 37 °C. Then isopropyl thiogalactoside (IPTG) was added to a final concentration of 1 mM. The cultures were incubated at 37 °C under vigorous shaking for additional 3 h and harvested by centrifugation.

Table 1: Different ELISA Protocols Used

format	steps of the assay
1	solid phase: BSA–Cs, Cyps, anti-Cyp-A Ab, second Ab–enzyme
2	solid phase: BSA–Cs, Cyps + Cs analogs, anti-Cyp-A Ab, second Ab–enzyme
3	solid phase: BSA–Cs, Cyps–Biotin, streptavidin–enzyme
4	solid phase: BSA–Cs, Cyps–Biotin + Cs analogs, streptavidin–enzyme
5	solid phase: BSA–Cs, Cyp-A–biotin + Cyps, streptavidin–enzyme

Purification of Recombinant Cyp-C. Cells (25 g wet weight) were resuspended in 100 mL of 50 mM Tris-HCl, pH 8.0, 5 mM benzamidine, 2 mM DTT, and 5 mM EDTA and lysed with a french press at 1200 bar. The suspension was then centrifuged at 35000*g* at 4 °C for 30 min, and the supernatant was discarded. The pellet was resuspended in 100 mL of lysis buffer and the whole process repeated twice. The procedure yielded about 4 g of crude inclusion bodies. The material was dissolved in 20 mL of 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM EDTA, and 5 M Gdn-HCl, and the suspension was clarified by centrifugation. The supernatant was then rapidly diluted in 1 L of 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM EDTA, and 0.5 M NaCl. The slightly cloudy solution was clarified by centrifugation for 30 min at 17000*g* (4 °C), and the volume was reduced to 3–4 mL by ultrafiltration with an Amicon YM 10 membrane. Contaminants and aggregates were removed from the soluble Cyp-C by a gel filtration step on a Pharmacia Superdex 75 Hiload column run at 2 mL/min in 20 mM Hepes, pH 7.4, and 100 mM NaCl. A single peak fraction containing soluble Cyp-C was collected, yielding 31 mg of protein, that migrated as a single species of predicted molecular weight in SDS–polyacrylamide gel electrophoresis and was homogeneous in HPLC.

Cyclophilin Biotinylation. Cyp-A, -B, and -C were biotinylated in 20 mM Hepes, pH 7.4, and 100 mM NaCl at variable concentrations (1–10 mg/mL), by adding 20 equiv of *N*-hydroxysuccinimide–biotin from a 100 mM stock solution in dimethyl sulfoxide and allowing the reaction to proceed, with gentle stirring, for 1 h at room temperature. The reactions were stopped by the addition to Tris-HCl, pH 7.8, to a final concentration of 50 mM (from a 1 M stock; hydrolysis of excess reagent). The samples were then gel-filtered on a Pharmacia Superdex 7S (HiLoad 16/60) equilibrated in 20 mM Hepes, pH 7.4, and 100 mM NaCl at a flow rate of 2 mL/min. The biotinylated Cyps were stored at 4 °C in the above buffer supplemented with 0.02% NaN_3 at a concentration of 0.1–0.2 mg/mL until use.

Anti-Cyclophilin Rabbit Antiserum. Rabbit antiserum directed against native Cyp-A purified from calf spleen was obtained as previously described (Ryffel et al., 1991). This antiserum detected both human and bovine Cyp-A with a detection limit of 10 ng/mL in ELISA (Quesniaux et al., 1987).

Enzyme Immunoassays. Five formats of solid-phase enzyme immunoassay (ELISA) were used (Table 1) to compare the direct binding of the Cyps to CsA (formats 1 and 3), to define the fine specificity of the Cyps for various CsA analogs (formats 2 and 4), and to determine the relative affinity of the Cyps (format 5). The assays were adapted from Quesniaux et al. (1987). Briefly, a D-Lys^8-Cs derivative was coupled to bovine serum albumin (BSA) and coated on a

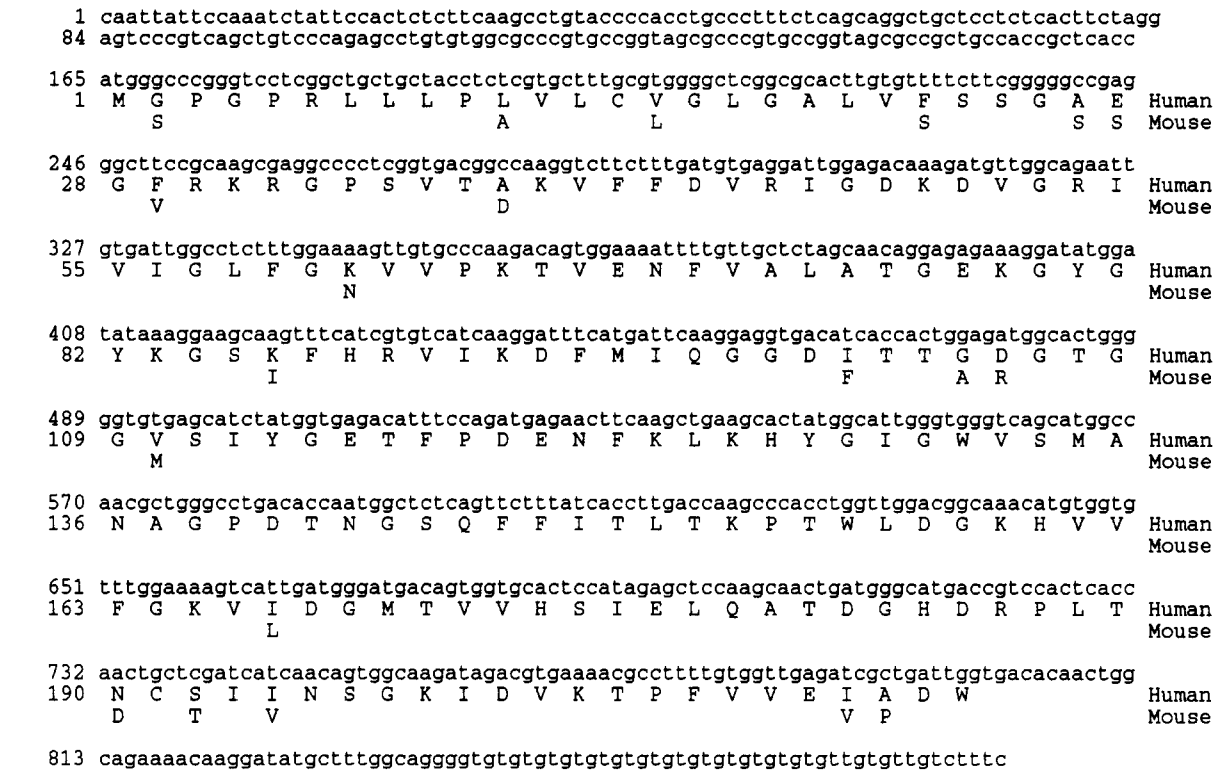

FIGURE 1: Nucleotide and deduced amino acid sequence of human cyclophilin C. Residues derived from the murine protein sequence are given where they are not identical to the human protein.

polyvinyl microtiter plate [2 μg/mL in phosphate-buffered saline (PBS), pH 7.4, for 2 h at 37 °C]. After saturation of the plate with 2% BSA in PBS (1 h at 37 °C) and washing once with 0.05% Tween 20 containing PBS (PBS-T) and three times with PBS, Cyp (formats 1 and 2) and Cyp–biotin (formats 3 and 4) were incubated overnight at 4 °C (in 1% BSA–PBS). In formats 1 and 2, anti-Cyp rabbit antiserum was then added (in 1% BSA–PBS for 2 h at 37 °C) and detected with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Sigma, St. Louis, MO; 1:1000 in 1% BSA–PBS, 2 h at 37 °C). In formats 3 and 4, bound Cyp–biotin was detected with streptavidin coupled to alkaline phosphatase (Jackson Immunoresearch Labs, Inc.; 1:6000 in 1% BSA–PBS, 2 h at 37 °C). The absorbance at 405 nm was measured after hydrolysis of *p*-nitrophenyl phosphate (1 mg/mL in diethanolamine buffer, pH 9.6, for 1–2 h at 37 °C).

In the competitive assays (formats 2 and 4), Cs derivatives (1 mg/mL ethanolic solution) were immediately added to the Cyp solutions (at 1:100 dilution, 0.83×10^{-5} M), and further 10-fold dilutions were made directly in the microtiter plate. After incubation overnight at 4 °C, unbound Cyp was removed and the assay continued as above. The competition obtained in the presence of Cs analogs was calculated as the percent inhibition of the control reaction between Cyp and coated Cs in the absence of inhibitor (10 replicates per plate). The IC_{50} for the Cs derivatives was compared with the IC_{50} for CsA run in triplicate on each microtitre plate. In format 5, increasing concentrations of unlabeled Cyp-A, Cyp-B, and Cyp-C used as competitor were added together with the Cyp-A–biotin conjugate, and the assay was continued as in format 3.

RESULTS

Cloning and Primary Structure of Human Cyclophilin C. A cDNA coding for human Cyp-C was isolated from a kidney cDNA library in phage λgt10 by hybridization with a murine Cyp-C probe spanning the coding region for amino acid residues 1–62 (Friedman & Weissman, 1991). We used this amino-terminal probe to minimize cross-hybridization with the abundant Cyp-A, as the sequence conservation is high in the central region of the Cyps. Approximately 600 000 phage plaques representing human kidney cDNA were screened under intermediate stringency conditions. Three weak and one strongly hybridizing phage were identified. Sequence analysis revealed that the less intense signals represented clones corresponding to Cyp-B. The fourth phage contained an insert of 883 base pairs and specified the entire coding region for human Cyp-C in analogy with the mouse counterpart. The nucleotide sequence and the deduced amino acid sequence are shown in Figure 1. Both mouse Cyp-C and human Cyp-C consist of 212 residues and share 90% amino acid identity. In both species, the amino-terminal region contains hydrophobic residues characteristic for signal sequences, which direct the protein to the endoplasmic reticulum. The amino acid homology to other well-characterized members of the human Cyp family, Cyp-A, Cyp-B, and Cyp-D, is also very high, as shown in Figure 2. Apart from the leader sequences, Cyp-C shares 70% identical residues with Cyp-B and has 48% homology with Cyp-A and Cyp-D. Moreover, all the residues in Cyp-A that, by X-ray crystallography, had been shown to form hydrogen bonds with CsA are conserved in Cyp-C, namely, Lys^{31}, Arg^{55}, Gln^{63}, Glu^{81}, Asn^{102}, Trp^{121}, and His^{126} (Pfügl et al., 1993).

Tissue Distribution. Total RNA from kidney, kidney-derived cell line HEK-293, and T cell line Jurkat was hybridized in parallel with radiolabeled full-length cDNA probes for Cyp-C and Cyp-A, respectively, used at identical concentrations of label. Signals corresponding to Cyp-C message (1.2 kb) and Cyp-A message (1.0 kb) were detected. The signal for Cyp-A was more than 10 times stronger than the signal for Cyp-C in the kidney and in HEK-293 cells (Figure 3). In Jurkat cells, Cyp-C mRNA was nearly absent,

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A    1                                                    mvnptvffdiaVD  GePLGR
                                                                      ||   |  ||||
D    1 MLalrcgsrwlgLLsvprsvplrlpaaracskgsgDpsssssgnplVYlDVDanGkPLGR
       ||          ||                          |             || |          ||
B    1 ML rlsernmkvLL aaaliagsVFflllpgpsAaDekKkGPkVTvKVYFDlRIGDeDVGR
                    |         ||         |    | || || || || |||| ||||
C    1    mgpgprlllpLvlcvglgalVF     ssgAegfrKrGPsVTaKVfFDvRIGDkDVGR

cons.     mla---g-r-l-ll---------vf-----s-ga-d--k-gpsvtvkvyfdv-ig----GR

                  *                              *       *
A   20 VsfELfADkVPKTAENFRALsTGEKGFGYKGScFHRiIPgFMCQgGDFTrHNGTGGKSIYG
       |  || || ||||||||||| |||||||||| ||| || |||| |||| ||||||||||
D   62 VvlELkADvVPKTAENFRALcTGEKGFGYKGStFHRVIPsFMCQaGDFTnHNGTGGKSIYG
       |   |    ||||  || || ||||||||| | |||||  || | ||||   |||||||||
B   60 VifGLFGKtVPKTVdNFVALATGEKGFGYKnSKFHRVIKDFMIQGGDFTrGDGTGGKSIYG
          ||||| ||||| |||||||||||| ||| ||||||||||||||| | |||||| ||||
C   54 iviGLFGKvVPKTVeNFVALATGEKGyGYKgSKFHRVIKDFMIQGGDiTtGDGTGGvSIYG

cons.     vvf-Lf--vVPKT-eNF-ALaTGEKGfGYKgSkFHRvI-dFM-QgGDfTr--GTGGkSIYG

          *                       *                   *     *
A   81 ekFeDENFiLKHtGPGiLSMANAGPNTNGSQFFICTaKTeWLDGKHVVFGkVKEGMniVea
         | |||| ||| ||| |||||||||||||||||| || |||||||||| |||||  |
D  123 sRFPDENFtLKHvGPGvLSMANAGPNTNGSQFFICTiKTdWLDGKHVVFGhVKEGMdVVkK
        ||||||| ||| |||  ||||||  |||||||| | || |||||||||| | ||| || |
B  121 ERFPDENFKLKHYGPGWVSMANAGkDTNGSQFFITTvKTaWLDGKHVVFGKVlEGMeVVrK
       | |||||||||||| ||||||||| |||||||||  |  ||||||||||||  || ||
C  115 EtFPDENFKLKHYGiGWVSMANAGpDTNGSQFFITltKptWLDGKHVVFGKVidGMtVVhs

cons.     erFpDENFkLKHyGpGw-SMANAGp-TNGSQFFI-t-Kt-WLDGKHVVFGkVkeGM-vV-k


A  142 mErFGSrnGkTSKKItI aDCGQLe
        | |||  | ||||| |  |||||
D  184 iESFGSkSGrTSKKIVI tDCGQLs
        ||    |     | ||  |||
B  182 vEStkTDSrDkPLKdVIIaDCGKIeVekPF  aIAke
        |   ||  | ||    |   ||| |  ||   ||
C  176 iElqaTDghDrPLtncsIinsGKIdVktPFvveIAdw

cons.     iEsfg-dsgdt--kiviiadcG--ev--pfvv-ia--
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FIGURE 2: Amino acid sequence comparison of human cyclophilins A, D, B, and C. Stars mark residues of Cyp-A, which have been shown by Pflügl et al. (1993) to interact with CsA in crystals.

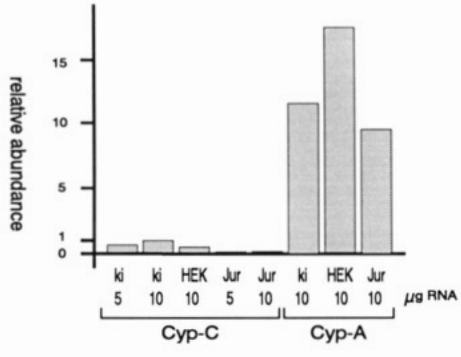

FIGURE 3: Comparison of Cyp-C and Cyp-A mRNA concentration by Northern blot analysis. Two filters with immobilized total RNA (5 or 10 μg) from kidney (ki), HEK-293 cells (HEK), and Jurkat cells (Jur) were hybridized with radiolabeled full-length probes of either Cyp-C or Cyp-A (10^6 cpm/mL of each). Signals were quantified and are given as relative activity compared to the signal measured for kidney RNA hybridized with Cyp-C.

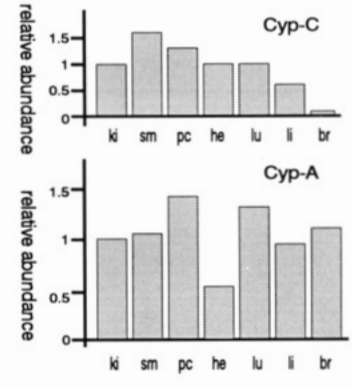

FIGURE 4: Tissue distribution analysis of human Cyp-C (upper panel) and Cyp-A (lower panel). A Northern blot containing 2 μg of poly-(A)–RNA of each kidney (ki), skeletal muscle (sm), pancreas (pc), heart (he), lung (lu), liver (li), and brain (br) was hybridized with a radiolabeled probe for Cyp-C and subsequently reprobed with Cyp-A. Signals were quantified by densitometry and are given as the expression in each tissue relative to that in kidney.

while Cyp-A was highly expressed. Stimulation of Jurkat cells with PMA ionomycin had no influence on Cyp-C mRNA levels (data not shown).

To further examine the tissue distribution of Cyp-C mRNA, Northern blots containing poly(A)–RNA from seven different human tissues were hybridized with a Cyp-C probe. We used an 172-bp fragment containing the 5′-untranslated sequence and the first 8 bp of the coding region as a probe to avoid cross-hybridization with Cyp-A, -B, and -D, as the 5′-untranslated regions show the least homology. Again, an mRNA of approximately 1.2 kb corresponding to Cyp-C was detected. In the tissues analyzed, the order of decreasing abundance of Cyp-C mRNA was skeletal muscle > pancreas > heart = kidney = lung > liver ≫ brain. The quantification of signals is given in Figure 4. Reprobing of the same filter with a full-length Cyp-A cDNA revealed the 1-kb Cyp-A transcript. The order of abundance of Cyp-A mRNA in these tissues was pancreas > lung > brain > skeletal muscle > kidney > liver > heart. However, the variation of Cyp-A mRNA levels in the different tissues was less than 3-fold, consistent with previous observations (Bergsma et al., 1991).

Table 2: Recognition by Cyp-A, Cyp-B, and Cyp-C of 36 Cs Derivatives Modified Singly at Each Amino Acid Residue

original residue	replacement residue	discrimination by cyclophilins[a] bovine Cyp-A[a]	human Cyp-A[b]	biotinylated human Cyp-A	Cyp-B	Cyp-C
MeBmt[1]	3′-deoxy	1.5	1	1.2	0.9	1.1
	3′-*O*-acetyl	1	>2	>2.5	2.1	>2.5
	7′-phenyl	0.3	0.4	0.1	0.2	0.3
	8′-hydroxy	0	0.3	−0.3	−0.2	0.1
	8′-phenylcarbonyl-oxydihydro	0	0.2	0.1	0	0.4
Abu[2]	Ser	0.7	0.7	0.7	0.6	0.7
	Ala	0.5	0.5	0.4	0.4	0.7
	Thr	0	0.1	−0.4	−0.4	−0.4
	aThr	0.5	0.6	0.6	0.6	0.2
	O-succinyl-Thr	2	1.7	1.5	1.2	1.2
	O-benzoyl-Thr	>2	>2	>2.5	2.5	>2.5
	Val	0.2	0.3	0.2	−0.6	−0.7
	DHVal	0.6	0.8	0.8	0.4	−0.3
	Nva	0.4	0.7	0	0.1	−0.3
Sar[3]	D-MeAla	−0.8	−0.7	−1.0	−0.4	−0.6
	D-MePhe	0	0.4	0.1	−0.1	0
	3′OH-D-MePhe	0.2	0.4	0	0.2	0
MeLeu[4]	MePhe	0.1	0.6	0.2	−0.6	−0.4
	γ-hydroxy-MeLeu	0	0	−0.3	0	−0.2
Val[5]	NVa[2], Nva	0.4	0.8	0.6	0.6	0.3
MeLeu[6]	MeAla	0.2	0.4	0.2	0.3	0.3
	MePhe	0.4	0.1	0.5	0.6	0.9
Ala[7]	Abu	0.3	0.1	0	−0.2	0
D-Ala[8]	D-Ser	−0.5	0.1	−0.5	−0.3	0
	D-Ser-(2-hydroxy-ethyl)	nd	0	−0.1	−0.3	−0.1
	D-Thr	−0.5	−0.1	0.2	0.1	0.4
	D-Lys	−0.4	−0.3	−0.8	−0.7	−0.4
	Boc-D-Lys	−0.3	−0.1	0	0.4	0.2
MeLeu[9]	MeAla	0.7	0.2	0.5	0.6	0.7
	MePhe	0.1	0.2	−0.4	0.1	0
MeLeu[10]	MeAla	1.2	0.7	1.4	1.3	1.2
	MePhe	0.5	0.4	0.5	0.4	0.3
MeVal[11]	MeIle	>2	2.0	1.8	2.3	2.0
	aMeIle	>2	>2	1.9	2.5	2.4
	MeLeu	>2	>2	>2	2.7	>2
	MeAla	0.9	0.9	0.8	0.9	1.0

[a] Results are expressed as the difference (in log) between the concentration of the Cs derivative and unmodified Cs required to achieve 50% inhibition in ELISA and represent the mean of two to four independent determinations. IC_{50} differences close to zero indicate that the modification in the particular Cs derivative does not influence its binding to Cyp. In contrast, IC_{50} differences of 1–2 log indicate that the substitution of the corresponding residue in the Cs molecule induced a 10–100-fold decrease of relative affinity and therefore that the original residue was involved in the interaction between Cs and Cyp. [b] Nonbiotinylated Cyp-A detected by means of antibodies. The IC_{50} of CsA for Cyp-A–biotin was similar to that determined for Cyp-A (≈3 × 10^{-8} M).

Expression and Purification. We generated a plasmid for the production of recombinant Cyp-C protein in *E. coli* using a T7 expression system (Studier et al., 1990). We choose alanine at coding position 19 as the beginning of the mature protein, according to the general characteristics of signal peptides (von Heijne, 1986). Cyp-C protein is produced only upon the IPTG induction of T7 polymerase in these strains and was recovered from inclusion bodies. From 25 g of *E. coli* we obtained nearly 30 mg of Cyp-C that migrated as a single species in SDS–PAGE (data not shown).

Affinity and Specificity of Binding of Cyp-A, -B, and -C to CsA. The interaction of Cyp-C with CsA was studied by immunochemical methods and compared to that of Cyp-A and Cyp-B. A Cs derivative was coupled to BSA via residue D-Lys[8] (BSA–D-Lys[8]-Cs), coated on a solid phase, and incubated with biotinylated Cyp-A, -B, and -C. Bound Cyp–biotin conjugates were detected with streptavidin coupled to alkaline phosphatase. To verify that biotinylation had no influence on Cyp affinity and fine specificity, control experiments were conducted with nonbiotinylated human Cyp-A

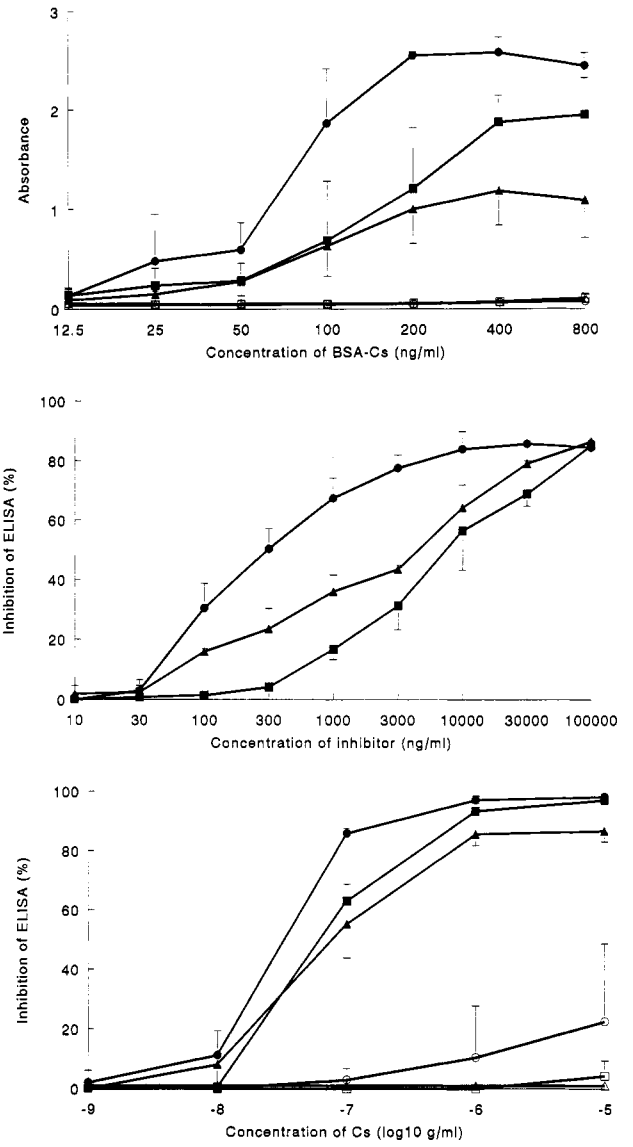

FIGURE 5: Recognition of cyclosporin by Cyp-A, Cyp-B, and Cyp-C in ELISA. (A, top) Binding of biotinylated Cyp-A (▲), Cyp-B (●), and Cyp-C (■) to BSA–D-Lys[8]-Cs (closed symbols) and BSA–Thr[2]-Cs (open symbols) coated on the solid phase. Bound Cyps were revealed with streptavidin coupled to alkaline phosphatase as described in Experimental Procedures. (B, middle) Competition by unlabeled Cyp-A (▲), Cyp-B (●), and Cyp-C (■) of the binding of biotinylated Cyp-A to BSA–D-Lys[8]-Cs. (C, bottom) Competition by CsA (closed symbols) and CsH (open symbols) of the binding of Cyp-A (▲), Cyp-B (●), and Cyp-C (■) to BSA–D-Lys[8]-Cs.

detected with anti-Cyp-A antibodies. Maximal binding to coated BSA–D-Lys[8]-Cs was obtained with similar concentrations of Cyp-A or Cyp-A–biotin (100 ng/mL). Biotinylation also had little influence on Cyp-A fine specificity (see below; Table 2).

All three proteins, Cyp-A, Cyp-B, and Cyp-C, were able to bind to BSA–D-Lys[8]-Cs but not to a conjugate of Cs coupled through Thr[2] to BSA, BSA–Thr[2]-Cs (Figure 5A). This gave a first indication that they recognized the same side of the Cs molecule, namely, that they bound to the face comprising residues 9–11 and 1–3, which is exposed when CsA is coupled through residue 8 but is buried when CsA is coupled through residue 2. The concentrations of Cyp-C required to achieve maximum binding to BSA–D-Lys[8]-Cs were 2–4-fold higher than that of Cyp-A or Cyp-B, indicating that the relative affinity of Cyp-C for CsA might be slightly lower than that of Cyp-A and Cyp-B. The differences in absolute signal levels

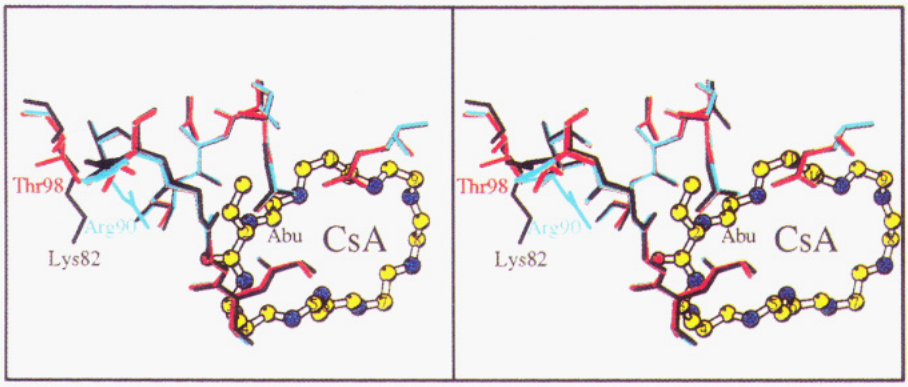

FIGURE 6: Stereoview of the Abu pocket in human cyclophilins A, B, and C complexed with CsA. Superposition of the Abu pocket of Cyp-A (black) on that of Cyp-B (cyan) and of Cyp-C (red). Human Cyp-A and Cyp-B are experimentally determined structures whereas human Cyp-C is a model. The only residue of Cyp-A which differs in sequence between Cyp-B and Cyp-C within this cavity is Lys^{82} (Arg^{90} in Cyp-B, Thr^{98} in Cyp-C). The Cα of Lys^{82} lies at about 10.0 Å of the Cβ of Abu^2. The backbone trace of CsA and the side chain of Abu^2 are displayed in ball and stick models (yellow for carbon, blue for nitrogen, and red for oxygen).

could reflect the different degree of biotinylation of the three Cyps.

The relative affinity of the three Cyps was further investigated in a competitive assay where the binding of biotinylated Cyp-A to BSA–D-Lys^8-Cs was competed by nonlabeled Cyp-A, Cyp-B, or Cyp-C. The best competitor was Cyp-B, followed by Cyp-A and by Cyp-C, indicating that, in this system as well, Cyp-B exhibited a higher relative affinity than Cyp-A, whereas the affinity of Cyp-C was the lowest (Figure 5B). These interactions were specific for native CsA since they could be completely inhibited by addition of free CsA but not of the nonimmunosuppressive CsH (D-$MeVal^{11}$-CsA), which exhibits a drastically distorted conformation compared to CsA (Walkinshaw et al., 1986). Fifty percent inhibition was obtained with (3–10) × 10^{-8} M CsA free in solution whereas only a slight inhibition was reached with 10^{-5} M CsH (Figure 5C).

Fine Specificity of Cyp-A, -B, and -C for CsA. The fine specificty of the interaction of natural bovine Cyp-A with CsA was previously determined using the ability of the Cyp-A to cross-react with a series of Cs derivatives, modified singly at each amino acid residue but showing a preserved conformation of the CsA peptidic skeleton as measured by ^{13}C NMR. CsA residues 1, 2, 10, and 11 were found to be essential for the binding to bovine Cyp-A (Quesniaux et al., 1987).

Using a similar approach, we studied the binding of human recombinant Cyp-A, Cyp-B, and Cyp-C to 36 Cs derivatives in a competitive ELISA where the binding of the biotinylated Cyps to BSA–D-Lys^8-Cs was competed by CsA or Cs derivatives. The results, summarized in Table 2, are expressed as the difference (in log) between the concentrations of the Cs derivative and of CsA required to achieve 50% inhibition of the control reaction without inhibitor (IC_{50}). Substitutions of CsA residues 3–9 had little or no influence on the interaction with Cyp-A, Cyp-B, or Cyp-C. Various substitutions of CsA residues 1, 2, 10, or 11 resulted in a strong decrease of binding to all Cyps, indicating that Cyp-A, -B, and -C recognize very similar epitopes on the CsA molecule. However, differences were observed in the recognition of Cs derivatives modified at residue Abu^2. Several derivatives were recognized better by Cyp-C than by Cyp-A or Cyp-B by comparison with CsA. This was the case for Val^2-CsA, which showed a slightly reduced affinity to Cyp-A compared to CsA but binds better to Cyp-B (4-fold) and Cyp-C (5-fold) than CsA does. DHVal^2-Cs, on the other hand, is recognized 2-fold better than CsA by Cyp-C but 6-fold and 2.5-fold worse by Cyp-A and Cyp-B, respectively.

DISCUSSION

While Cyp-A is a ubiquitous protein and is believed to be the target protein involved in CsA-derived immunosuppression, murine Cyp-C was reported to be expressed in a restricted subset of tissues relative to Cyp-A and Cyp-B with relatively high levels in the kidney (Friedman & Weissman, 1991; Bram et al., 1993; Ke et al., 1993). Hence, Cyp-C was suggested to be involved in CsA effects on kidney. We have determined the relative levels of human Cyp-C mRNA compared to Cyp-A in Jurkat cells and kidney. The Jurkat cell line, which is generally used to study T cell activation, was nearly free of Cyp-C transcript while Cyp-A was highly expressed. In human kidney, Cyp-C mRNA was detected, but it was at least 10-fold less abundant than Cyp-A. This is in agreement with the fact that, out of approximately 600 000 recombinant phages obtained from a human kidney cDNA library, only one single clone was positive for Cyp-C. Simultaneous isolation of three Cyp-B clones with extended 5′-untranslated regions makes it unlikely that this was due to underrepresentation of the 5′-ends. We could detect Cyp-C mRNA in similar to higher concentrations also in other tissues which are not affected by CsA *in vivo*, e.g., skeletal muscle. Therefore, our data obtained from human tissues do not support a specific role for Cyp-C in CsA nephrotoxicity which was proposed on the basis of the earlier studies of its relative abundance in the mouse kidney.

The binding of the recombinant Cyp-A, -B, and -C to CsA and a series of 36 Cs derivatives indicates that, overall, the sites recognized by the Cyps on CsA are very similar. This is not surprising, considering the extensive sequence homology between these proteins. All three Cyps are unable to bind to solid-phase-coated Cs conjugated to BSA through residue 2, when residues 10–2 are buried and thus not available for interaction. Replacement of Abu^2 by Val^2 in CsA lead to an increase of binding affinity to Cyp-C and, to a lesser extent, to Cyp-B but not to Cyp-A. Another derivative, DHVal^2-Cs, is less well recognized than CsA by Cyp-A and Cyp-B, while Cyp-C recognized it better than CsA. Therefore, the Abu^2 residue *per se* is not essential for the high-affinity interaction of Cyp-C with CsA. According to the crystal structure of the CsA–Cyp-A complex (Pflügl et al., 1993), the nitrogen of Abu^2 forms a hydrogen bond with the carbonyl oxygen of Asn^{102} of Cyp-A, which is conserved in Cyp-C as well as in Cyp-B and Cyp-D. Therefore, it is possible that the conformation of Cyps around the direct contact points might also be relevant for the recognition of CsA.

Recently, the crystal structure of murine Cyp-C has been published (Ke et al., 1993). Human Cyp-C has 95% identity

with murine Cyp-C in the central core of the protein, i.e., between residues 26 and 166. Amino acids which differ in sequence between human and murine Cyp-C are at a distance of greater than 9 Å from the CsA binding site and are solvent exposed. This suggests that the conformation of the CsA binding site of human Cyp-C might be essentially similar to that of murine Cyp-C (Ke et al., 1993) and accordingly to that of Cyp-A (Mikol et al., 1993).

A comparison of the CsA binding site between human Cyp-A and human Cyp-C indicates no differences except in the region where the Abu^2 residue of CsA binds (the Abu pocket) where a lysine (Lys^{82} in Cyp-A) is replaced by a threonine (Thr^{98} in Cyp-C), thus generating more space in the Abu pocket (Figure 6). This enlarged Abu pocket can account for the higher tolerance of Cyp-C for modification of CsA in position 2. In the case of Cyp-B, this Lys^{82} is replaced by an Arg^{90}, which folds back into the pocket, thus reducing its size (Mikol et al., 1994).

The reduced binding of $DHVal^2$-Cs and Val^2-Cs by Cyp-A correlates with their reduced immunosuppressive effects, as measured *in vitro* (G. Zenke, unpublished results). However, these Cs derivatives bind better to Cyp-C than CsA. These results indicate that Cyp-A is possibly a more relevant target than Cyp-C for immunosuppression, which is consistent with the extremely low expression of Cyp-C mRNA in Jurkat cells. Both derivatives might be useful to study the specific role of Cyp-C in cell physiology. As Cyp-C is generally expressed at a 10-fold lower level compared to Cyp-A, Cyp-C could be saturated preferentially to other Cyps by these derivatives. In particular, the compound $DHVal^2$-Cs could be a very useful reagent as it also binds less to Cyp-B. Two other immunosuppressive cyclosporins used in the clinic, Nva^2-Cs (CsG) and D-Ser-(2-hydroxyethyl)^8-Cs (IMM125), have similar binding to Cyp-A, -B, and -C than CsA (see Table 2).

The 77-kDa glycoprotein CyCAP has been identified and shown to bind to murine Cyp-C but not to Cyp-A and Cyp-B (Friedman et al., 1993). As CsA inhibits CyCAP interaction with Cyp-C, it was proposed that CyCAP and CsA share a similar binding site on Cyp-C with subtle differences to Cyp-A and Cyp-B. Although we find alterations in the fine specificity interaction with Cs derivatives, they are not sufficient to fully explain the exclusive CyCAP–Cyp-C interaction. However, CyCAP might mimic the structure of $DHVal^2$-Cs and thus interacts with high affinity to Cyp-C compared to Cyp-A and Cyp-B. Alternatively, the CyCAP interaction with Cyp-C might include residues additional to those binding to CsA and which have a different conformation in Cyp-A and Cyp-B.

The isolation of human target proteins and analysis of their role in cellular physiology are important exercises in the understanding of the molecular mechanism of CsA action. It is hoped that this information will lead to rational identification of novel therapeutic agents.

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