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An Endoplasmic Reticulum-Specific Cyclophilin

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Cyclophilin is a ubiquitously expressed cytosolic peptidyl-prolyl *cis-trans* isomerase that is inhibited by the immunosuppressive drug cyclosporin A. A degenerate oligonucleotide based on a conserved cyclophilin sequence was used to isolate cDNA clones representing a ubiquitously expressed mRNA from mice and humans. This mRNA encodes a novel 20-kDa protein, CPH2, that shares 64% sequence identity with cyclophilin. Bacterially expressed CPH2 binds cyclosporin A and is a cyclosporin A-inhibitable peptidyl-prolyl *cis-trans* isomerase. Cell fractionation of rat liver followed by Western blot (immunoblot) analysis indicated that CPH2 is not cytosolic but rather is located exclusively in the endoplasmic reticulum. These results suggest that cyclosporin A mediates its effect on cells through more than one cyclophilin and that cyclosporin A-induced misfolding of T-cell membrane proteins normally mediated by CPH2 plays a role in immunosuppression.

Cyclosporin A (CsA) is a lipophilic cyclic undecapeptide initially isolated from the fungus *Trichoderma polysporum* (4) and found to be a potent immunosuppressive drug (25). Primarily applied in organ transplantation, CsA use must be carefully monitored due to severe side effects, such as nephrotoxicity (44). Biochemical and pharmacological effects of CsA on cultured cells have been described (2, 5, 27), the most striking of which is inhibition of interleukin-2 mRNA expression in T cells (10, 32). This inhibition appears to be caused by CsA acting indirectly on several transcription factors needed for activation of interleukin-2 mRNA expression (11, 39). Whether CsA has other relevant effects at the molecular level is unknown.

A search for an intracellular receptor for CsA resulted in the discovery of cyclophilin (CPH), a 17-kDa cytosolic proteins that binds CsA with high affinity (22, 23). Cytosolic CsA-binding activity has been demonstrated for every eukaryotic organism examined (29), and in vertebrates, CPH mRNAs are expressed in virtually all tissue types (8). CPH and the protein-folding enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) from pig have been shown to be identical (14, 53), and CsA inhibits the PPIase activity of pure human CPH samples (36). Treatment of living chicken embryo tendon fibroblasts with CsA inhibits the proper folding of procollagen I, indicating that PPIase activity is a physiological function of CPH (51). The link between inhibition of PPIase by CsA treatment and immunosuppression is not yet evident.

mRNAs for CPH have been cloned from many species, and their sequences have been determined (3, 8, 19, 24, 35); a tremendous amount of conservation exists among the encoded proteins. In lower eukaryotes, multiple forms of CPH have been described. For instance, in *Saccharomyces cerevisae*, two distinct forms of CPH have been described (9, 20, 30), and in *Neurospora crassa*, one gene is differentially transcribed into two mRNAs which encode cytosolic and mitochondrial forms of CPH (54). The *ninaA* gene of *Drosophila melanogaster* encodes an eye-specific CPH (48, 49). Southern blots examining rat (8) and human (19) genomic DNA with CPH cDNA probes identify multiple bands, suggesting that a multigene family for CPH exists. At the protein level, 80% of CsA binding to extracts made from murine thymoma cells was to cytosolic CPH (42), while the remaining CsA was found associated with membrane fractions, suggesting that CPH-related proteins exist. Accordingly, we used a degenerate oligonucleotide probe to isolate mouse and human cDNA clones of an evolutionarily conserved mRNA encoding a CPH-related protein and demonstrated that it binds CsA, has PPIase activity, and, unlike CPH, is located in the endoplasmic reticulum (ER). These findings suggest that PPIase activities are necessary in many cell compartments and indicate that CsA treatment may affect multiple cellular processes.

MATERIALS AND METHODS

Isolation of mouse and human cDNA clones. Filters containing 20,000 plaques of a mouse thymus cDNA library constructed in lambda ZAP (Stratagene Cloning Systems) were screened with ^{32}P-radiolabeled KH1, a degenerate oligonucleotide (TGGC/TTGGAC/TGGNAAGCAC/TGTG GT). Hybridization was done in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–0.2% NaH_2PO_4–5× Denhardt's solution–10 mM EDTA–100 μg of salmon sperm DNA per ml–100 μg of yeast tRNA per ml at 42°C for 15 h, and the filters were then washed successively in 6× SSC–0.1% SDS at room temperature, 37, 42, and 52°C for 30 min each. The filters were exposed to XAR-5 film (Kodak) for 16 h and developed. Positive bacteriophage plaques were converted into plasmid by in vivo excision repair (50), and small-scale plasmid preparations were performed. Sequences were determined (47) with KH1 and KH3 (an oligonucleotide complementary to KH1) as sequencing primers. Sequences not corresponding to the mouse CPH1 sequence (24) were searched against the GenBank and EMBL data banks (Intelligenetics). Two clones were apparently independent cDNA isolates of the same mRNA, and one of these was used to further screen both 200,000 plaques from the mouse thymus library, from which 18 positives were isolated (one full-length), and 200,000 plaques from a human thymus library (Stratagene Cloning Systems), from which 16 positives were isolated, the longest of which was included in this study.

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Northern (RNA) blot hybridization analysis. Total RNA and poly $(A)^+$ RNA were isolated from mouse and rat tissues (1, 18), separated by electrophoresis on formaldehyde-agarose gels, and transferred to nitrocellulose membranes by standard methods (38). Radioactive probes were prepared by the random primer method (12), and blots were hybridized by standard methods (38).

DNA sequencing. Double-stranded DNA sequencing (47) was performed with T7 polymerase (Pharmacia) and oligonucleotides synthesized to correspond to regions spaced approximately every 200 bp. The 5′ end of the mouse sequence was confirmed by direct RNA sequencing (16), using mouse liver poly $(A)^+$ RNA as the template.

Construction of bacterial expression vectors with mouse cDNA clones. The protein-coding region of mouse clone pMU6.0 was amplified by the polymerase chain reaction (46) with either of two 5′-end-specific primers and one 3′-end-specific primer. Primer 1 (CCACGTCATATGCTGCGCCT CTCGGAGCG) and primer 3 (CCATTAGAATTCCCCCAG GCTCTCTACTCC) were used to synthesize the complete open reading frame starting at nucleotide 16 (see Fig. 2). Primer 2 (CCGAACCACATATGAACGATAAGAAGAAG GGACCTA) and primer 3 were used to synthesize the coding sequence of the predicted mature protein (after cleavage of a putative signal sequence). The polymerase chain reaction-amplified fragments were digested with *Nde*I and *Eco*RI and then ligated into the T7 RNA polymerase expression vector pRK172 (40). The construct containing the predicted mature protein-coding sequence (pCPH2E6) was transformed into *Escherichia coli* BL21(DE3)(pLysS) (52).

Purification of recombinant mouse CPH2 protein. A 2-liter culture of *E. coli* BL21 (DE3)(pLysS) (52) containing the CPH2 expression plasmid pCPH2E6 was grown (37°C) to an optical density at 600 nm of 0.5 in LB broth containing ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the cells were grown for a further 2 h. Cells were harvested by centrifugation, resuspended in 60 ml of cell lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg each of aprotinin, leupeptin, and pepstatin per ml) and lysed by two passages through a French press at 10,000 lb/in^2. Cell debris was removed by centrifugation (14,000 × *g*, 15 min). Solid $(NH_4)_2SO_4$ was slowly added to the supernatant to a final concentration of 70%, and then the mixture was allowed to stand for 1 h at 4°C. After centrifugation (14,000 × *g*, 15 min), the supernatant was dialyzed against TE (20 mM Tris-HCl [pH 8.0], 1 mM EDTA) with frequent buffer changes. The dialyzed extract was batch absorbed to DE-52 anion-exchange resin (Whatman) that had been preequilibrated with TE. Nearly pure CPH2 was recovered in the supernatant fraction after removal of DE-52 resin and concentrated in an Amicon ultrafiltration cell (P10 membrane).

CsA binding and PPIase assay. CsA binding assays were done with ^{3}H-CsA (Amersham) by the Sephadex LH-20 (Pharmacia) column assay method previously described (22). PPIase activity was assayed as previously described (13, 36).

Antibody preparation and purification. Bacterially expressed CPH2 was purified from cell lysates as described above, and the proteins remaining in the DE-52-depleted supernatant were precipitated by the addition of 20% trichloroacetic acid and incubation at 0°C for 15 min. Following centrifugation at 14,000 × *g* for 10 min, the pellet was solubilized by the addition of SDS sample buffer (2% SDS, 150 mM Tris-HCl [pH 8.8], 10% sucrose, 10 mM dithiothreitol) and incubation at 95°C for 5 min. The proteins were then separated by preparative SDS-15% polyacrylamide gel electrophoresis by the method of Laemmli (33). Following electrophoresis, the proteins were transferred to 0.22-μm-pore-size nitrocellulose membranes (Schleicher & Schuell) by the method of Burnette (6), and the major band corresponding to CPH2 was identified by straining with ponceau S. The CPH2 band was cut from the nitrocellulose sheet, solubilized with dimethyl sulfoxide, and diluted with an equal volume of complete Freund adjuvant. Rabbits (New Zealand White) were initially immunized by subcutaneous injection with approximately 300 to 500 μg of CPH2 in the dimethyl sulfoxide-Freund adjuvant solution. The animals were boosted 4 weeks later with an additional 300 to 500 μg of CPH2 prepared as described above except that following solubilization of the nitrocellulose, the dimethyl sulfoxide was diluted with an equal volume of 5-mg/ml alum. The animals were bled 10 to 14 days following each boost, and the sera were screened by immunoblot analysis of bacterial extracts from CPH2- or CPH1-expressing cells using ^{125}I-protein A and autoradiography as the detection method.

Antibodies were purified from reacting sera with a CPH2 affinity column prepared as described previously (17). The affinity column was made by immobilization of purified CPH2 to CNBr-activated Sepharose CL-4B (Pharmacia) at a ratio of 5 mg of CPH2 per 1 ml of beads, using the manufacturer's recommended protocol. Approximately 0.1 to 0.2 mg of affinity-purified antibody was recovered from 10 ml of sera, and the antibodies were routinely stored at 4°C in phosphate-buffered saline (10 mM sodium phosphate [pH 7.4], 140 mM NaCl, 0.2% sodium azide).

Cell fractionation of mouse and rat liver. Fractionation of mouse and rat liver into cytosolic and membrane fractions was done as previously described (15). Fractions were resolved by SDS-polyacrylamide gel electrophoresis (33) and analyzed by Western blot (immunoblot) (6) with affinity-purified antibodies specific for mouse CPH2. Monoclonal anti-ribophorin II was provided by C. Crimaudo (7), and polyclonal serum specific for α-1,2-mannosidase II was provided by S. Pind (Scripps Clinic, La Jolla, Calif.).

GenBank accession number. The accession numbers for the sequences reported here are M60456 (pMU6.0) and M60457 (pHU28).

RESULTS

Isolation of clones of mouse and human CPH2 mRNAs. Molecular and biochemical evidence suggested that more than one CPH-related protein exists in vertebrate cells. The amino acid sequences of known CPHs were compared, and a degenerate oligonucleotide, KH1, corresponding to a highly conserved amino acid sequence, WLDGKHVV, was synthesized and used to screen a phage cDNA library made in lambda ZAP from mouse thymus mRNA. Twenty-six positive clones surviving secondary screens were converted into plasmids by in vivo excision, and partial sequences were determined. The sequences of the majority of the cDNA clones encoded a protein 97.6% identical to rat CPH and thus corresponded to mouse CPH (24). The sequences of two isolates putatively encoded a novel distinct CPH-related protein.

Total RNA from mouse brain, liver, kidney, and heart was isolated and analyzed by Northern blotting with the 800-bp cDNA insert from one of the novel clones as a probe (Fig. 1). An RNA species of approximately 1.0 kb was detected in each of the four samples, and the probe did not cross-

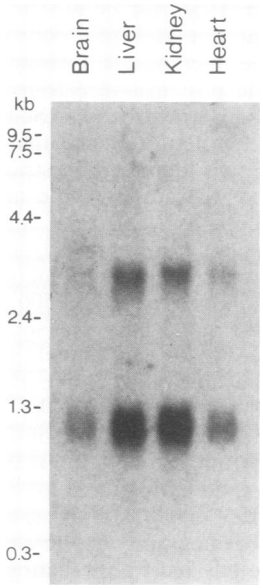

FIG. 1. Northern blot analysis of a novel CPH-related RNA. Total RNA (20 μg per lane) from mouse brain, liver, kidney, and heart was fractionated by electrophoresis and Northern blotted with a mouse CPH2 probe. Shown on the left are the mobilities of markers whose lengths in kilobases are given. The RNA with slower mobility may represent a precursor.

hybridize with the 0.85-kb mRNA encoding CPH. The mouse cDNA library was rescreened with the novel cDNA insert, and a longer clone (pMU6.0) was isolated, which subsequent primer extension analysis (data not shown) with mouse liver poly(A)$^+$ RNA indicated was a full-length isolate of one of a family of mRNAs with heterogeneous transcription start sites. A human thymus library was screened in parallel, and the cDNA clone with the largest insert (pHU28) was selected for further study. The 800-bp insert was also used as a probe on a Southern blot that contained mouse genomic DNA digested with restriction endonuclease *Eco*RI, *Hin*dIII, or *Bam*HI. The probe detected only one band in each case, suggesting that the gene for this mRNA is single copy (data not shown).

Sequence and expression of CPH2. The nucleotide sequences of the mouse cDNA clone pMU6.0 and the human cDNA clone pHU28 were determined, and each contains a single open reading frame (Fig. 2). The mouse sequence is 873 nucleotides long, possesses two in-frame AUGs near its 5′ end, and is about 65% identical to known mammalian CPH mRNA sequences. If translation were to start at the second AUG, which resembles more closely the consensus translation initiation sequence proposed by Kozak (31), a 208-amino-acid protein would be synthesized. The first 25 amino acids of this putative protein resemble a signal sequence (55) with a predicted cleavage occurring between Ala-25 and Asn-26. Such a cleavage would result in a 20-kDa final product. The encoded putative protein sequence has 64% identity to mouse CPH (24), with the maximum similarity found in the middle third of the protein sequence. Given this pronounced similarity, we adopted the convention of calling the original CPH CPH1 and the novel molecule described here CPH2.

The nucleotide sequence of the human CPH2 homolog (Fig. 2) extended only to the G of the second AUG found in the mouse sequence. Its open reading frame terminated at exactly the same point as the mouse sequence (Fig. 2). The encoded protein was 94% identical to the mouse CPH2 sequence over 207 compared amino acids. Effective alignment of the 3′ untranslated mouse and human sequences required introduction of 12 gaps, while no gaps were necessary for aligning the open reading frames.

Poly(A)$^+$ RNA was prepared from 34 different rat tissues and analyzed in series by Northern blotting to compare the expression of CPH2 mRNA and CPH1 mRNA (Fig. 3). Steady-state levels of CPH2 mRNA were usually, although not always, lower than CPH1 mRNA levels, but CPH2 mRNA was detectable in all tissues examined. The levels of CPH2 mRNA expression in liver, intestine, and placenta were about 10-fold higher than CPH2 mRNA levels in other tissues. The CPH2/CPH1 ratio was highest in the epididymis and diaphragm and lowest in the eye, testis, brain, thymus, and spleen.

A transcription vector was constructed that included mouse CPH2 sequences, starting with nucleotide 16 and ending at nucleotide 680, cloned downstream from the T7 RNA polymerase promoter. RNA was synthesized in vitro and translated in vitro with a rabbit reticulocyte lysate in the presence or absence of microsomal membranes. When the cell-free translation products were resolved by SDS-polyacrylamide gel electrophoresis, a 22-kDa doublet was observed (data not shown), in agreement with the predicted sizes for proteins whose translation initiated at either of the first two AUGs of the nucleotide sequence. With the addition to the translation system of microsomal membranes capable of cleaving signal peptides, a polypeptide with a relative mobility of 20 kDa was observed (data not shown). These results support the notion that a signal sequence is present on CPH2 and that signal peptidase cleaves at or near Ala-25.

Functional analysis of bacterially expressed CPH2. To investigate the nature of the CPH2 protein, we constructed a second expression plasmid for the purpose of preparing large quantities of pure CPH2 protein. The coding sequence for the predicted mature CPH2 protein (residues 26 to 208) was fused to an initiator methionine codon downstream from a T7 promoter, and this plasmid was transformed into an IPTG-inducible T7 RNA polymerase expression host (52). Upon induction with IPTG, a major protein of 20 kDa was observed by Coomassie blue staining of bacterial extracts separated by SDS-polyacrylamide gel electrophoresis (Fig. 4a). The induced protein was isolated in nearly pure form (Fig. 4a) from lysed cells by its solubility in 70% ammonium sulfate and nonaffinity for an anion-exchange resin. Microsequencing of the N-terminal end of the purified protein gave the sequence MNDKK (data not shown), indicating that the initiator methionine was retained, followed by the expected CPH2 amino acids. Purified CPH2 was found to be very stable in 20 mM Tris-HCl (pH 7.5) at 4°C.

The purified protein was assayed for CPH1-like activities. Binding of ^{3}H-CsA to purified CPH2 was found to be saturable, with an apparent K_d = 400 nM (Fig. 4b), comparable to the binding constant (K_d = 200 nM) measured for purified bovine CPH1 (22). Extracts from bacteria lacking the CPH2 expression plasmid showed no binding (data not shown). PPIase activity was examined by using racemization of an *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate as an assay (34). Purified CHP2 protein accelerated the conversion of the chymotrypsin-resistant *cis* isomer to the chymotrypsin-sensitive *trans* isomer, showing that CPH2 is a PPIase (Fig. 4c). The conversion rate depended on CPH2

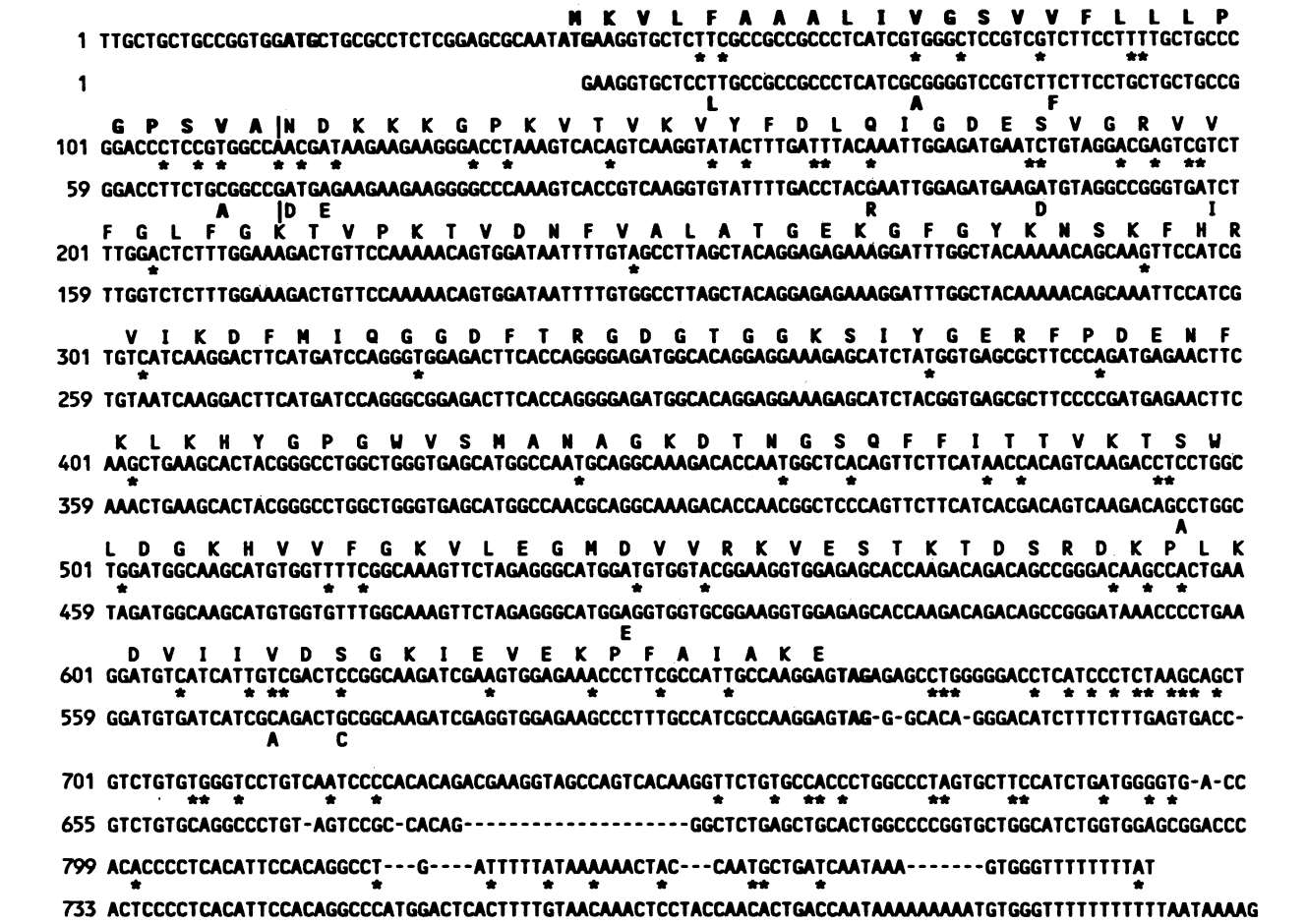

FIG. 2. Nucleotide sequence of mouse and human CPH2. The nucleotide sequences of the mouse CPH2 clone pMU6.0 (top) and the human clone pHU28 (bottom) are aligned; asterisks indicate nucleotide differences between the two sequences. The deduced complete mouse amino acid sequence is shown above the mouse nucleotide sequence; differences in the deduced human amino acid sequence are indicated below the human nucleotide sequence. The mouse protein sequence shown begins at the second in-frame methionine. The first 25 amino acids of mouse CPH2 that resemble a signal peptide sequence (55) are shown in boldface. The vertical lines represent the predicted site of signal peptidase cleavage.

concentration, and CsA addition completely inhibited the catalytic activity of CPH2 (Fig. 4c).

Cellular localization of CPH2. An antiserum was raised in rabbits against purified bacterially expressed CPH2, and high-affinity anti-CPH2 antibodies were isolated by affinity chromatography. The affinity-purified antibodies did not cross-react with bacterially expressed mouse CPH1, whose mobility on gels was faster than that of CPH2 (data not

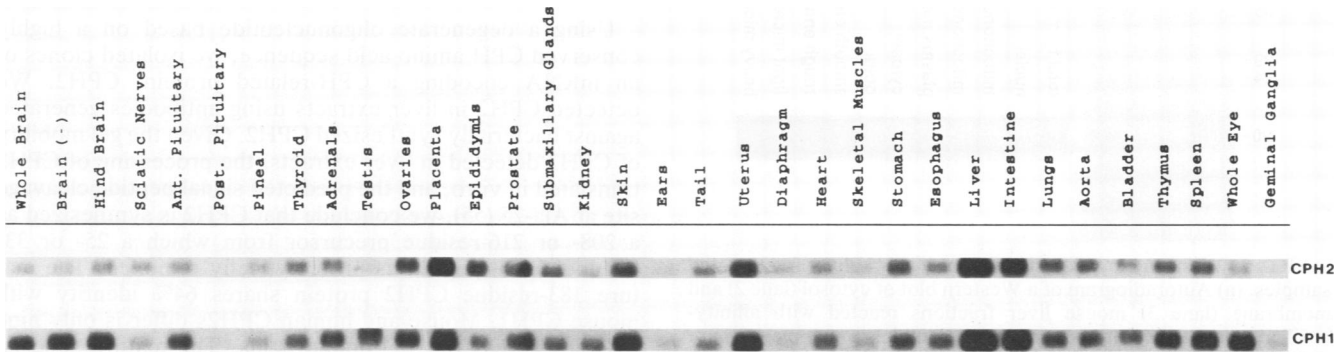

FIG. 3. Northern blot analysis comparing CPH1 and CPH2 mRNA expression. Northern blotted poly(A)$^+$ RNA purified from the 34 indicated rat tissues was sequentially hybridized with radioactively labeled probes representing rat CPH1 (8) and mouse CPH2. The CPH1 and CPH2 probes detected poly(A)$^+$ RNA with mobilities of 0.85 and 1.0 kb, respectively. Shown is a composite of the Northern blots with comparable probe-specific activities and equal exposure times. Only the hybridizing regions of the blots are shown.

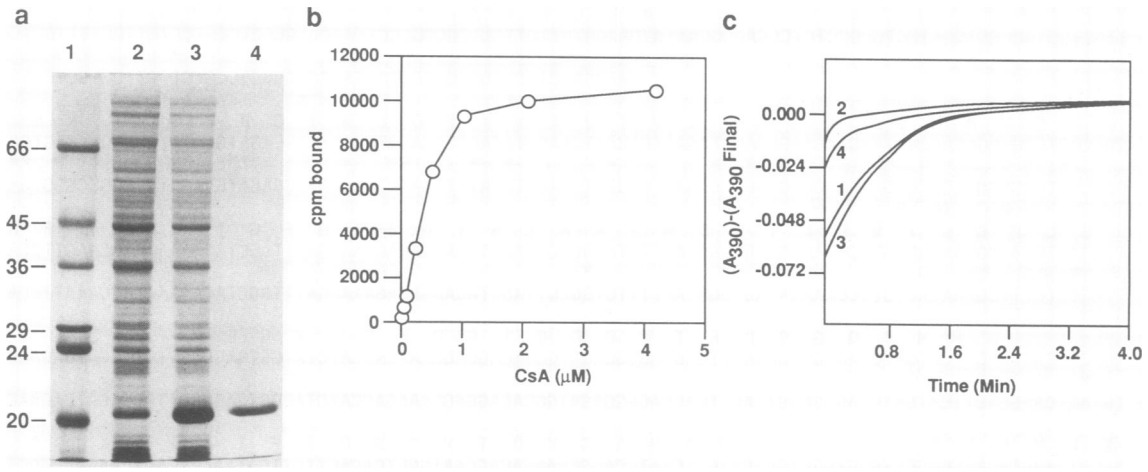

FIG. 4. Bacterially expressed CPH2 binds CsA and has PPIase activity. (a) Coomassie blue-stained SDS-12% polyacrylamide gel containing the following: lane 1, protein standards with their respective sizes (in kilodaltons) indicated on the left; lanes 2 and 3, total bacterial lysates before the addition of IPTG (lane 2) or after 2 h of incubation with 0.4 mM IPTG (lane 3); lane 4, sample of purified CPH2 used for CsA binding and PPIase assays. (b) Binding of ^{3}H-CsA to purified CPH2 protein. The concentration of CPH2, estimated by the Bradford method, was constant (12.5 μM), while the concentration of added ^{3}H-CsA was varied. If the protein concentration is valid, then only 10 to 20% of the recombinant CPH2 is active. (c) PPIase activity of purified CPH2 and its inhibition by CsA as measured by the chymotrypsin-coupled chromogenic assay. Curves were redrawn from original dot-matrix plots of spectrophotometric data. Curves: 1, no CPH2 added; 2, 0.6 μM CPH2; 3, 0.6 μM CPH2 + 5 μM CsA; 4, 0.3 μM CPH2.

shown). This CPH2-specific affinity-purified antibody was used to examine cytosolic and membrane fractions of a mouse liver homogenate by Western blot analysis. The affinity-purified antibody specifically recognized a 20-kDa protein displaying the same relative gel mobility as purified bacterially expressed CPH2. The immunoreactive protein was present in the membrane fraction, and none was detected in the cytosol fraction (Fig. 5a), even with longer exposures (data not shown). The location of CPH2 contrasts with the exclusive cytosolic location of CPH1 (29, 41, 42).

To examine the subcellular distribution of CPH2 in more detail, we fractionated rat liver and examined the fractions by Western blot analysis. High CPH2 levels were found in all four ER fractions examined (smooth and rough ER and light and heavy microsomes), while much lesser amounts of CPH2 were found in the plasma membrane and Golgi fractions and no CPH2 was detected in the cytosolic, mitochondrial, and nuclear fractions. Previous work with ER-specific enzymatic markers (15) demonstrated that these enzymes were detectable in these same fractions at about the same ratios that we observe for CPH2. To verify the validity of our rat liver fractionation, we analyzed by immunoblot these same fractions with antibodies to the ER-specific protein ribophorin II and the Golgi-specific protein mannosidase II and found the expected distribution for these marker proteins (data not shown). When the affinity-purified antibody was used to examine cultured NIH 3T3 cells by immunofluorescence, no discernible cellular structures were detected (data not shown), possibly due to an inability of the affinity-purified antibody to recognize native CPH2 protein.

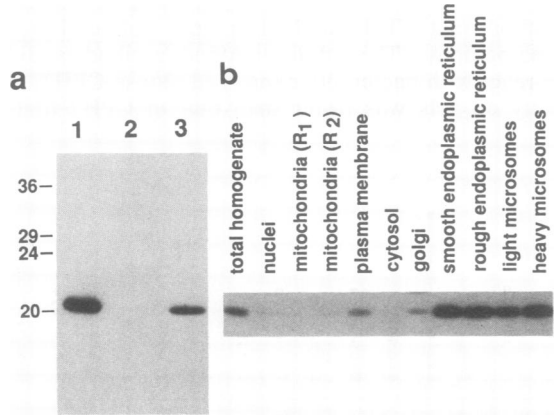

FIG. 5. Western blot analysis of mouse and rat liver fractionated samples. (a) Autoradiogram of a Western blot of cytosol (lane 2) and membrane (lane 3) mouse liver fractions reacted with affinity-purified anti-CPH2 antibody. Lane 1 contains a sample of pure bacterially expressed CPH2 protein (Fig. 4a, lane 4), and on the left the mobilities of standard proteins used as molecular size markers are indicated (in kilodaltons). (b) Autoradiogram of Western blot analysis of rat liver subfractionated samples (50 μg of protein per lane) reacting against affinity-purified anti-CPH2 antibodies.

DISCUSSION

Using a degenerate oligonucleotide based on a highly conserved CPH amino acid sequence, we isolated clones of an mRNA encoding a CPH-related protein, CPH2. We detected CPH2 in liver extracts using antibodies generated against bacterially synthesized CPH2. Given the gel mobility of CPH2 detected in liver extracts, the processing of CPH2 translated in vitro, and the predicted signal peptide cleavage site at Ala-25 (55), we conclude that CPH2 is synthesized as a 208- or 216-residue precursor from which a 25- or 33-residue signal peptide is proteolytically removed. The mature 183-residue CPH2 protein shares 64% identity with mouse CPH1. Mouse and human CPH2s differ is only nine residues across the 183 amino acids. The mature species, which is highly enriched in the ER, binds CsA (K_d = 400 nM) and has CsA-inhibitable PPIase activity. Thus, CPH2 is a member of the CPH family. The 1.0-kb CPH2 mRNA was detected in every tissue we examined, although most tissues

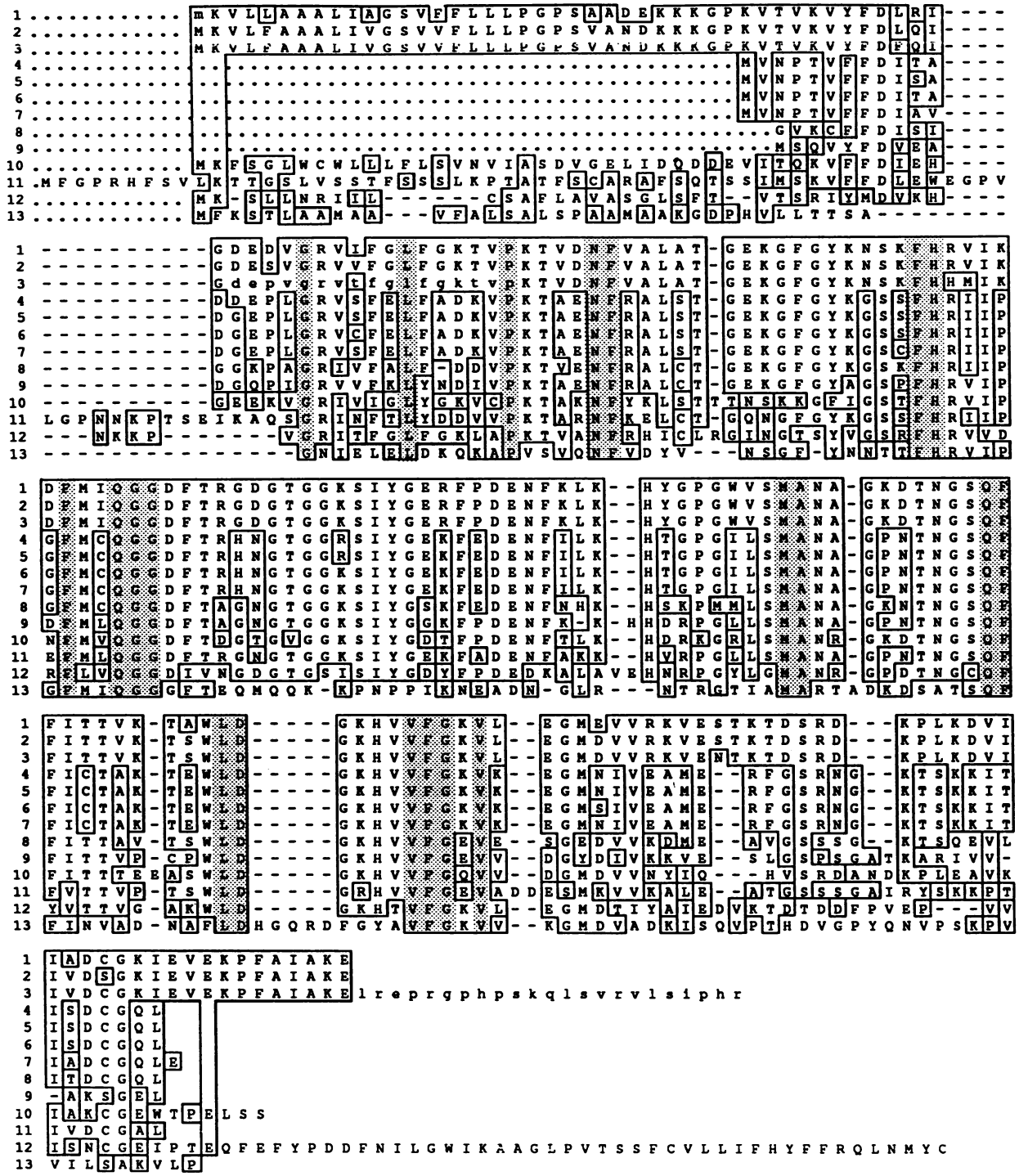

FIG. 6. Alignment of CPH-related proteins. Thirteen CPH protein sequences derived from cloned cDNAs were aligned. Sequences are numbered on the left and are as follows: 1, human CPH2 (this study); 2, mouse CPH2 (this study); 3, rat CPH related (26); 4, mouse CPH1 (24); 5, hamster CPH1 (3); 6, rat CPH1 (8); 7, human CPH1 (19); 8, *Echinococcus granulosus* CPH (35); 9, *S. cerevisae* CPH1 (9, 20); 10, *S. cerevisae* CPH2 (30); 11, *N. crassa* CPH1/2 (54); 12, *Drosophila* CPH (48, 49); 13, *E. coli* CPH (28). Any two or more amino acids that match vertically are boxed, and in all places where an amino acid is conserved among all 13 proteins, the row is shaded. We believe, based on the alignment of our mouse and human CPH2 proteins, that the reported DNA sequence of a rat CPH-related protein (26) that we found to be inexactly homologous to the conserved CPH2 sequence contains a few frameshift errors between amino acids 46 and 62. If the frameshifts are corrected, the amino acids shown in lowercase in the figure match almost perfectly with human and mouse sequences, thus suggesting that the protein is a rat homolog of CHP2. The rat sequence also continues past the terminator in the human and mouse sequences for 23 additional amino acids: again, we represent these in lowercase, pending closer examination of the putative rat CPH2 sequence.

contained less CPH2 mRNA than CPH1 mRNA. Only the epididymis and diaphragm contained more CPH2 mRNA than CPH1 mRNA. The global expression of CPH2 mRNA suggests that CPH2 PPIase activity is necessary in the ER of all cell types.

We aligned the sequences of the 13 known CPH-related proteins (Fig. 6), including that of a bacterial CPH-related protein (28) recently shown to be a non-CsA-binding PPIase (37). Twenty-one amino acids of approximately 160 aligned were absolutely conserved among all 13 sequences. If the bacterial sequence was removed from the comparison, an additional 26 amino acids were conserved. The highest conservation was observed in the central portion of the CPH-related sequences, while the two ends have seen more change. This suggests that the PPIase enzymatic active site is in the middle of the molecule and that the ends represent regions of the protein that have adopted other functional roles. Removal of the bacterial sequence from the alignment closed the gap imposed at the sequence WLDGKHVVFG (the sequence used to synthesize the degenerate oligonucleotide KH1). The tryptophan residue at the beginning of this amino acid string has been shown to be important for CsA binding (22), which suggests that conservation of this sequence is necessary to maintain a proper binding pocket for CsA. Another feature of the mouse CPH2 sequence is the absence of cysteine residues, yet the protein has PPIase activity, supporting the hypothesis that cysteines are not involved in the enzymatic mechanism (37).

Recently, CsA was found to bind to and inhibit a mitochondrial matrix peptidyl-prolyl *cis-trans* isomerase (21), which raised the question of whether CPH2 was equivalent to this protein. Our immunochemical data show that CPH2 is located almost exclusively in the ER, not the mitochondria. This leads us to speculate that a third, yet undiscovered, mitochondrion-specific CPH is present in vertebrate cells. Indeed, each cellular compartment may have its own specialized CPH. CPH2 has neither the ER retention sequence KDEL (43) nor possible long membrane-spanning domains, raising a question as to how CPH2 is retained within the ER. Perhaps it is anchored as a component of a complex whose function is to fold or otherwise modify proteins traveling through the ER.

Our results demonstrate that there is more than one cellular target for CsA inhibition. This adds to the overall complexity of the cascade of biochemical events that CsA treatment of cells probably initiates but suggests that all affected events derive from a reduction in the level of appropriately conformed proteins. The inhibition of a PPIase in the ER may reduce the number of correctly folded proteins that eventually travel to the surface of the cell. In T cells, improperly folded cell surface receptors will probably affect the immune response. Therefore, we consider it a plausible scenario that CsA prevents graft rejection by inhibiting CPH2.

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ADDENDUM

While this manuscript was being reviewed, another group published the cloning and expression of human CPH2 (45). A comparison of their sequence with ours (pHU28) showed two silent nucleotide differences in the open reading frame and four differences at the extreme 3′ end of the sequence.

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