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Immunology

Cloning and characterization of cyclophilin C-associated protein: A candidate natural cellular ligand for cyclophilin C

(cyclophilin/cyclosporin A/proline isomerase/signal transduction)

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ABSTRACT **We report the protein purification and the cloning and characterization of a cDNA encoding the proteins that bind with high affinity to cyclophilin C (Cyp-C) in the absence of cyclosporin A. Transfection of this cDNA into COS cells directs the production of a glycoprotein of 77 kDa that binds to Cyp-C in the absence, but not the presence, of cyclosporin A. Homology comparisons reveal that this protein and gene, termed CyCAP for Cyp-C-associated protein, possess a cysteine-rich domain (scavenger receptor cysteine-rich domain) found in a variety of cell-surface molecules; the rest of the sequence is apparently specific. This result raises the possibility that Cyp-C serves as a mediator or regulator of an as-yet-unidentified signal or cellular process initiated via the Cyp-C-associated protein.**

Cyclophilins are a family of proteins that bind the immunosuppressive drug cyclosporin A (CsA) and that possess cis/trans peptidylprolyl isomerase activity (1, 2). To date at least four types of mammalian cyclophilins have been cloned, cyclophilins A, B, C (Cyp-A, Cyp-B, and Cyp-C, respectively), and hCyP3 (3–7). In addition, cyclophilin homologues have been isolated from a variety of invertebrate species, including *Drosophila* (8), *Neurospora* (9), and *Saccharomyces* (10, 11). In spite of this wide distribution, the physiological functions of cyclophilins have yet to be elucidated. Only the *Drosophila* cyclophilin homologue *ninaA* has been implicated in a particular activity in cells in which it is expressed (12, 13). NinaA appears to be involved in the proper processing of a specific type of rhodopsin. Both the ninaA and the mammalian B cyclophilins are localized to the endoplasmic reticulum. Such a localization supports the hypothesis that these cyclophilins have a potential role in protein folding. In contrast, Cyp-A appears to be free in the cytosol of cells (1). Cyp-C can be found in the secretory pathway, as well as on the surface of viable cells (M.T., unpublished work).

Recent work suggests that isolated Cyp-A, Cyp-B, and Cyp-C possess similar properties in the presence of the drug CsA, as all three cyclophilins can form a complex *in vitro* with calcineurin, a calcium-dependent serine/threonine phosphatase (6, 14, 15). Several groups have provided evidence to support the hypothesis that inhibition of calcineurin is the key biochemical step in mediating the immunosuppressive activities of the drugs CsA and FK506 (14–20). In the absence of CsA, the properties of these mammalian cyclophilins diverge. Cyp-C interacts with high affinity with at least four cellular proteins of molecular masses of 77 kDa, 60 kDa, 37 kDa, and 25 kDa, whereas Cyp-A and Cyp-B do not. The interaction of Cyp-C with these associating proteins can be disrupted or prevented by the addition of saturating amounts of CsA.

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When considering the physiological role of cyclophilins, one presumes that the interactions these proteins experience in the absence of exogenous compounds like CsA reflect their normal functions. Thus, molecules such as the 77-, 60-, 37-, and 25-kDa proteins may help clarify the role(s) cyclophilins are designed to play in organisms from yeast to higher eukaryotes. Here we demonstrate by protein sequence analysis that the 77-, 60-, and 37-kDa proteins share peptides in common. Molecular cloning of the cDNA that encodes these sequences reveals an open reading frame of 574 amino acids that directs the production of a glycoprotein of 77 kDa.†

MATERIALS AND METHODS

Cell Culture, Harvesting, and Protein Purification. AC.6, a mouse bone-marrow-derived stromal cell line, was grown as described (6). Confluent AC.6 cells were washed with cold phosphate-buffered saline and lysed on ice with Triton X-100 lysis buffer, as described (6). Supernatants, precleared with 6 μg of glutathione-*S*-transferase (GST) and 25 μl of glutathione agarose (Sigma) per ml of lysate (10 hr at 4°C), were then incubated with 2 μg of Cyp-C–GST and 25 μl of glutathione agarose per ml of lysate (1–3 hr at 4°C). The beads were washed with lysis buffer, and proteins were eluted with 2 vol of 50 mM Tris·HCl (pH 6.5) plus CsA at 30 μg/ml for 15–30 min on ice. Trichloroacetic acetate-precipitated samples were separated on either 9.5% or 11% SDS/PAGE gels. After electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes (Millipore) and stained with Coomassie blue; bands of interest were then excised for further processing.

Preparation of Proteins for Sequence Determination. *In situ* protease digestion and peptide microsequencing were done, as described (21–23) with the following modifications: polyvinylidene fluoride membranes containing the proteins were blocked with 0.5% polyvinylpyrrolidone-40/methanol for 1 hr before trypsin digestion in 100 mM Tris·HCl, pH 8.5/5% (vol/vol) acetonitrile at a substrate/enzyme ratio of 20:1 (wt/wt) at 37°C overnight. Peptides were separated by reversed-phase HPLC and subjected to automated Edman degradation.

Isolation of cDNA Clone. Oligonucleotides (degenerate and/or inosine containing) were synthesized for several stretches of protein sequence and used in PCR reactions in all possible pairwise combinations. The template for PCR reactions was plasmid DNA from AC.6 cell cDNA libraries (6). Standard PCR buffer with 1.5 mM $MgCl_2$ was used for all reactions (24). The PCR conditions were as follows: 94°C for 1 min, 37°C for 2 sec, 72°C for 1 min for 30 cycles. No PCR

Abbreviations: CsA, cyclosporin A; Cyp-A, Cyp-B, and Cyp-C, cyclophilins A, B, and C, respectively; CyCAP, Cyp-C-associated protein; SRCR domain, scavenger receptor cysteine-rich domain; GST, glutathione-*S*-transferase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L16894).

products were detectable after 30 cycles of amplification. A 0.5-μl aliquot of this reaction mixture was further amplified as follows: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles. PCR products were cloned and ligated into modified pBluescript SK(−) plasmid vectors (25) and sequenced by using the dideoxynucleotide chain-termination method (26) and Sequenase reagents (United States Biochemical). The successful primer pair was as follows: sense primer (with *Bam*HI site), 5′-CCGGATCC **GTI GA(G/A) ATI TT(C/T) TA**-3′; antisense primer (with *Eco*RI site), 5′-CAGAATTCA **AA IGT IGG (G/A)TC (C/T)TG IGG**-3′.

A full-length cDNA clone was obtained by screening an AC.6 cDNA library (6) with the PCR clone, essentially as described (27). ^{32}P-labeled probes were prepared by random priming the PCR fragment. Colony screening was repeated until isolated positive colonies were obtained and screened by PCR using primers internal to the primers used in isolating the original 664-bp partial cDNA clone.

Homology searches were done by using the FASTDB program of IntelliGenetics software. Sequence alignments and protein structure analysis were done by using the GENALIGN and PEP programs from Intelligenetics, and the PROSITE program (28).

RNA Analysis. RNA analysis was done, as described (6). Labeled probe was prepared as described above by using the 664-bp partial cDNA isolated by PCR.

Transfection Experiments. The Cyp-C-associated protein (CyCAP)-coding region was cloned into a modified pSR α vector (ref. 29, from Dan Denney, Stanford University). COS cells were transfected with 10 μg of pSR-α or pSR-α–CyCAP plasmid DNAs, and 72-hr post-transfection cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, essentially as described (6). Cyp-C–GST affinity reactions were done as described (6) by using 5×10^6 cpm per reaction. For the glycosidase experiment, labeled CyCAP bound to Cyp-C–GST–glutathione agarose was treated with endoglycosidase F/*N*-glycosidase F, as described by the manufacturer (Boehringer Mannheim). Samples were analyzed by 10% SDS/PAGE.

RESULTS

Identification and Purification of CyCAPs. The gene for Cyp-C was isolated from a subtracted cDNA library from the bone-marrow-derived stromal cell line AC.6 (6). Initial experiments using recombinant Cyp-C protein as an affinity reagent to probe labeled AC.6 cell extracts revealed that in the absence of CsA a protein of ≈77 kDa bound. When CsA was present during the incubation, this 77-kDa protein was no longer bound, and a calcineurin–calmodulin complex was bound (6, 14). Cyp-C–GST affinity purification of unlabeled AC.6 cell extracts revealed the 77-kDa protein and additional bands in the absence of CsA. Fig. 1 demonstrates the spectrum of AC.6 cell lysate proteins bound to either GST or Cyp-C–GST and eluted by CsA. The proteins eluted specifically from Cyp-C–GST by CsA have apparent molecular masses of 77, 60, 37, and 25 kDa; the three smaller proteins run as diffuse bands (lanes 3 A and B).

The 77-, 60-, and 37-kDa Proteins Share Peptides. Table 1 demonstrates the amino acid sequence relationship among the 77-, 60-, and 37-kDa proteins. Protein sequence from the ≈25-kDa protein has not been obtained, as this protein comigrates with significant contaminating bands on SDS gels. The 77-, 60-, and 37-kDa proteins contain common tryptic peptides, suggesting that all species arise from a common precursor, possibly the 77-kDa protein. In addition, an antibody raised against the 77-kDa protein recognizes the 60- and 37-kDa proteins in immunoblotting experiments, showing the presence of a common epitope (M.T., unpublished observation).

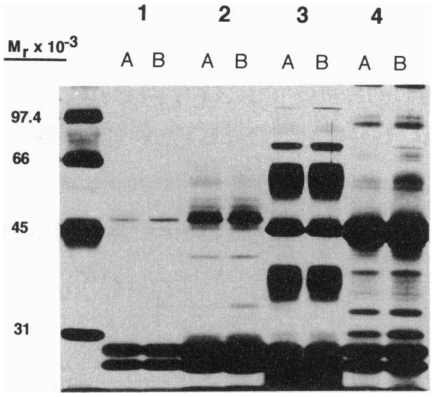

FIG. 1. AC.6 proteins bound to a Cyp-C affinity column and elution by CsA. Silver-stained SDS/PAGE gel demonstrates AC.6 lysate proteins bound to GST affinity matrix and eluted with CsA (control) (lanes 1 A and B). Proteins remaining on GST affinity matrix after CsA elution are released by boiling in sample buffer (control) (lanes 2 A and B). Proteins bound to Cyp-C–GST affinity matrix are eluted with CsA (lanes 3 A and B). Proteins remaining on Cyp-C–GST affinity matrix after CsA elution are released by boiling in sample buffer (lanes 4 A and B). All experiments were run in duplicate. Lanes 3 A and B show proteins of 77 kDa, 60 kDa, 37 kDa, and ≈25 kDa, which bind specifically to Cyp-C and are eluted by addition of CsA. The doublet at ≈26 kDa in all lanes is GST, whereas the protein of 45 kDa in lanes 3 and 4 is the Cyp-C–GST fusion protein.

Cloning the cDNA Encoding Cyp-C-Associated Proteins. Data from several regions of protein sequence were used to generate both degenerate and/or inosine-containing oligonucleotides. The AC.6 cDNA library was used as the template for the PCR reactions (6). PCR amplification proved to be successful in isolating a partial cDNA that was used as a probe to isolate longer cDNA clones from the AC.6 library. Several clones were isolated, and the sequence of the longest clone is presented in Fig. 2. An additional partial clone isolated from a cDNA library prepared from the EL-4 murine T-cell line was also sequenced and found to be identical in the region of overlap with the sequence in Fig. 2.

The isolated CyCAP cDNA is 2176 nt long and encodes a distinctive protein of 574 amino acids with a predicted molecular mass of 64 kDa and a predicted unmodified pI of 4.7. The cDNA possesses a 5′ untranslated region of 175 nt and a 3′ untranslated region of 276 nt. Other features of CyCAP that are evident from sequence analysis are as follows: five potential N-linked glycosylation sites (residues circled in Fig. 2), several potential phosphorylation sites for protein kinase C and casein kinase-2 and potential myristoylation sites (data not shown). The N terminus of CyCAP consists of uncharged and largely hydrophobic residues (underlined in Fig. 2), which serve as a signal sequence, as CyCAP possesses N-linked sugars (see Fig. 5*C*). Protein sequences shown in italics in Fig. 2 correspond to peptides sequenced from purified protein before isolation of the cDNA.

Homology searches at the amino acid level reveal that CyCAP contains a cysteine-rich region, the scavenger recep-

Table 1. Peptides generated by microsequencing

Peptide	Sequence
77-kDa 1	**MQALEF**
60-kDa 1	**TMQALEF**HTVPVEVLAK
37-kDa 1	**TMQALEF**HTVPVEVLAK
60-kDa 2	**AV?QMSTEEIA**
37-kDa 2	**AV?QQSTEEIA**
60-kDa 3	VEIFYR
60-kDa 4	ALGYE?ATQALGR
60-kDa 5	LFAT?QDPTFIT

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CAGCCTCGTC CCAGGAAAGC CACATGGTGG AGCTTTTCTC TGCTGAACAG TCTACAGAAG GCTTCCAGTT GCAGCCAGGA AGGAGGCTCC
AGGACTGCCT TCAGCCACTG CCTCGCTGCC TGAGGGACAG TTGAGTCCCT CTCTTGCTCC CAGGGTTGGG CTTCTAGGCC AGGCA

176  ATG GCT CTC CTG TGG CTC CTC TCT GTG TTC TTG CTG GTT CCA GGG ACT CAA GGT ACA GAA GAT GGA GAC ATG
  1  MET Ala Leu Leu Trp Leu Leu Ser Val Phe Leu Leu Val Pro Gly Thr Gln Gly Thr Glu Asp Gly Asp MET

248  GGC TTG GTT AAC GGG GCC TCA GCC AAT GAG GGC CGC GTG GAG ATC TTC TAC AGA GGC CGG TGG GGG ACA GTG
 25  Gly Leu Val Asn Gly Ala Ser Ala Asn Glu Gly Arg Val Glu Ile Phe Tyr Arg Gly Arg Trp Gly Thr Val

320  TGT GAC AAC CTC TGG AAC CTT TTG GAT GCC CAC GTC GTC TGC CGG GCC CTG GGC TAT GAG AAC GCC ACC CAA
 49  Cys Asp Asn Leu Trp Asn Leu Leu Asp Ala His Val Val Cys Arg Ala Leu Gly Tyr Glu Asn Ala Thr Gln

392  GCA CTG GGC AGA GCT GCC TTC GGG CCA GGA AAG GGA CCG ATC ATG CTG GAT GAG GTG GAA TGT ACA GGG ACC
 73  Ala Leu Gly Arg Ala Ala Phe Gly Pro Gly Lys Gly Pro Ile MET Leu Asp Glu Val Glu Cys Thr Gly Thr

464  GAG TCC TCA CTG GCC AGT TGC AGA TCC CTG GGT TGG ATG GTG AGC CGC TGT GGG CAC GAG AAG GAC GCA GGC
 97  Glu Ser Ser Leu Ala Ser Cys Arg Ser Leu Gly Trp MET Val Ser Arg Cys Gly His Glu Lys Asp Ala Gly

536  GTG GTC TGC TCC AAC GAT ACC ACG GGG CTT CAC ATC CTG GAC CTC TCT GGA GAG CTC TCA GAT GCA CTG GGC
121  Val Val Cys Ser Asn Asp Thr Thr Gly Leu His Ile Leu Asp Leu Ser Gly Glu Leu Ser Asp Ala Leu Gly

608  CAG ATC TTT GAC AGC CAG CAG GGC TGC GAC CTA TTC ATC CAG GTG ACA GGG CAG GGG TAT GAG GAC CTG AGC
145  Gln Ile Phe Asp Ser Gln Gln Gly Cys Asp Leu Phe Ile Gln Val Thr Gly Gln Gly Tyr Glu Asp Leu Ser

680  CTC TGT GCC CAC ACG CTG ATC CTG CGC ACC AAC CCC GAG GCC CAG GCC CTG TGG CAA GTG GTG GGC AGC AGC
169  Leu Cys Ala His Thr Leu Ile Leu Arg Thr Asn Pro Glu Ala Gln Ala Leu Trp Gln Val Val Gly Ser Ser

752  GTC ATC ATG AGA GTG GAT GCT GAG TGC ATG CCT GTC GTC AGA GAC TTC CTC AGG TAC TTT TAC TCC CGA AGA
193  Val Ile MET Arg Val Asp Ala Glu Cys MET Pro Val Val Arg Asp Phe Leu Arg Tyr Phe Tyr Ser Arg Arg

824  ATC GAG GTC AGC ATG TCT TCT GTT AAG TGC TTG CCA AAG CTA GCC TCT GCC TAT GGG GCC ACA GAG CTT CAG
217  Ile Glu Val Ser MET Ser Ser Val Lys Cys Leu Pro Lys Leu Ala Ser Ala Tyr Gly Ala Thr Glu Leu Gln

896  GAC TAC TGT GGA CGG CTT TTT GCC ACC CTC CTC CCC CAA GAC CCC ACT TTC CAT ACT CCC TTG GAC CTT TAT
241  Asp Tyr Cys Gly Arg Leu Phe Ala Thr Leu Leu Pro Gln Asp Pro Thr Phe His Thr Pro Leu Asp Leu fyr

968  GCG TAC GCA CGG GCC ACC GGG GAC TCT ATG CTG GAA GAT CTG TGT GTA CAG TTT CTG GCC TGG AAC TTC GAG
265  Ala Tyr Ala Arg Ala Thr Gly Asp Ser MET Leu Glu Asp Leu Cys Val Gln Phe Leu Ala Trp Asn Phe Glu

1040 CCT CTG ACA CAG TCT GAG TCC TGG TCG GCT GTT CCC ACC ACC TTG ATC CAG GCT CTC CTC CCC AAG AGT GAG
289  Pro Leu Thr Gln Ser Glu Ser Trp Ser Ala Val Pro Thr Thr Leu Ile Gln Ala Leu Leu Pro Lys Ser Glu

1112 CTG GCT GTG TCT AGT GAG CTG GAT CTG CTG AAG GCA GTG GAC CAG TGG AGC ACA GAA ACC ATT GCC TCA CAC
313  Leu Ala Val Ser Ser Glu Leu Asp Leu Leu Lys Ala Val Asp Gln Trp Ser Thr Glu Thr Ile Ala Ser His

1184 GAG GAT ATA GAG CGC CTG GTG GAA CAG GTC CGC TTC CCC ATG ATG CTG CCC CAG GAG CTG TTC GAG CTG CAG
337  Glu Asp Ile Glu Arg Leu Val Glu Gln Val Arg Phe Pro MET MET Leu Pro Gln Glu Leu Phe Glu Leu Gln

1256 TTC AAC CTG TCC TTG TAC CAA GAT CAC CAG GCA CTG TTC CAG AGG AAG ACC ATG CAG GCC TTG GAG TTC CAC
361  Phe Asn Leu Ser Leu Tyr Gln Asp His Gln Ala Leu Phe Gln Arg Lys Thr MET Gln Ala Leu Glu Phe His

1328 ACA GTG CCT GTC GAA GTG CTG GCC AAG TAC AAA GGC CTG AAC CTC ACG GAG GAC ACC TAC AAG CCC CGC CTT
385  Thr Val Pro Val Glu Val Leu Ala Lys Tyr Lys Gly Leu Asn Leu Thr Glu Asp Thr Tyr Lys Pro Arg Leu

1400 TAC ACC TCT TCC ACC TGG AGT AGC TTG GTG ATG GCC TCC ACC TGG AGG GCA CAA AGA TAT GAA TAC AAT CGA
409  Tyr Thr Ser Ser Thr Trp Ser Ser Leu Val MET Ala Ser Thr Trp Arg Ala Gln Arg Tyr Glu Tyr Asn Arg

1472 TAC AAT CAG CTC TAT ACA TAT GGC TAT GGC TCA GTA GCC CGG TAC AAT AGC TAC CAG TCC TTC CAA ACC CCA
433  Tyr Asn Gln Leu Tyr Thr Tyr Gly Tyr Gly Ser Val Ala Arg Tyr Asn Ser Tyr Gln Ser Phe Gln Thr Pro

1544 CAA CAC CCC AGC TTC CTC TTC AAG GAC AAC GAG ATC TCC TGG TCA GCC ACC TAC CTC CCC ACC ATG CAG AGC
457  Gln His Pro Ser Phe Leu Phe Lys Asp Asn Glu Ile Ser Trp Ser Ala Thr Tyr Leu Pro Thr MET Gln Ser

1616 TGC TGG AAC TAT GGC TTC TCG TGT ACC TCT AAC GAG CTC CCT GTA CTG GGC CTC ACC ACA TCC AGC TAC TCC
481  Cys Trp Asn Tyr Gly Phe Ser Cys Thr Ser Asn Glu Leu Pro Val Leu Gly Leu Thr Thr Ser Ser Tyr Ser

1688 AAT CCG ACA ATT GGC TAT GAG AAC AGA GTA CTG ATC CTC TGC GGA GGC TAC AGT GTG GTG GAT GTC ACC AGC
505  Asn Pro Thr Ile Gly Tyr Glu Asn Arg Val Leu Ile Leu Cys Gly Gly Tyr Ser Val Val Asp Val Thr Ser

1760 TTT GAA GGC TCC AAG GCC CCT ATT CCC ACT GCC CTG GAC ACC AAT AGT TCC AAG ACT CCC TCC CTC TTT CCC
529  Phe Glu Gly Ser Lys Ala Pro Ile Pro Thr Ala Leu Asp Thr Asn Ser Ser Lys Thr Pro Ser Leu Phe Pro

1832 TGT GCC TCA GGG GCC TTT AGC AGC TTC CGT GTG GTC ATA CGC CCC TTC TAC CTC ACT AAC CTC CAC TGA
553  Cys Ala Ser Gly Ala Phe Ser Ser Phe Arg Val Val Ile Arg Pro Phe Tyr Leu Thr Asn Leu His  .

1901          CATGGTGTAA ATGGTACATC TCAGTGGTGG GGACGCAGAC ATTCCTGTGT CCCCTCCTTC GCCTCCAGCT GCTTTGTAAG
   CATAAACTGA TTGTATTCAA ATGAAAATTT ACTAGAAGGT TTCAGCCAGC ACTCACTCCA GGACTGAGAG TCCCAGGGCC CTCACTGCAG
   GTACAAGCAG GTTCCATGAG GTCCTGTGGG ATTTCCTGTG CCTACTGCAG TAGCCCCATC TGTCACAGTC ACTCATCAAA AATCATTAAA
   GTCTCACGTG CTTCTC   2176
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FIG. 2. Nucleotide sequence of CyCAP cDNA and protein sequence of CyCAP. CyCAP cDNA is 2176 nt long and encodes a protein of 574 amino acids, with a predicted molecular mass of 64 kDa. The 5′ untranslated region is 175 nt long, and the 3′ untranslated region is 276 nt long. Circled residues are sites of potential N-linked glycosylation. A hydrophobic region at the N terminus that serves as a signal sequence is underlined. Protein sequences presented in italics were independently derived by sequencing of purified protein fragments.

tor cysteine-rich (SRCR) domain, found in the extracellular region of several cell-surface proteins and in secreted proteins (30–36). This domain spans ≈100 amino acids and occurs just after the signal sequence near the N terminus of CyCAP. The SRCR proteins with the greatest amino acid identity with CyCAP in this region are bovine macrophage scavenger receptor type I (32), 54%; the ninth SRCR domain of bovine WC1.1 protein (36), 53%; mouse macrophage scavenger receptor type I (31), 52%; human macrophage scavenger receptor type I (33), 47%; and the first SRCR domain of the sea urchin speract receptor (35), 36%. Although many members of this family of proteins possess multiple copies of this cysteine-rich domain, CyCAP has only a single copy. The macrophage scavenger receptor type I, which also has a single copy of the SRCR domain, forms a trimeric protein. If CyCAP is a multimeric protein, it is not through disulfide linkages, as unreduced samples have the same apparent molecular mass as reduced samples when analyzed by SDS/PAGE (M.T., unpublished observation). An alignment of the SRCR domains of bovine macrophage scavenger receptor, WC1.1 protein, and the speract receptor with CyCAP is shown in Fig. 3. The amino acids shared among all four proteins (in boldface type) matches the consensus SRCR domain (31).

Fig. 4 shows an RNA analysis of CyCAP mRNA expression. Labeled CyCAP cDNA hybridizes to a single message of ≈2400 nt. CyCAP is expressed by AC.6 bone marrow

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mCyCAP    26  LVNGASANEGRVEIFYRGRWGTVCDNLWNLLDAHVVCRALGYENATQAL
bMSR     356  LVGGSGPHEGRVEIFHEGQWGTVCDDRWELRGGLVVCRSLGYKGVQSVH
bWC1.1   934  LVDGGGPCGGRVEILDQGSWGTICDDDWDLDDARVVCRQLGCGEALNAT
Speract   45  LIHGRTENEGSVEIYHATRWGGVCDWWWHMENANVTCKQLGFPGARQFY

mCyCAP    75  GRAAFGPGKGPIMLDEVECTGTESSLASCRSLGWMVSR-CGHEKDAGVVC
bMSR     405  KRAYFGKGTGPIWLNEVFCFGKESSIEECRIRQWGVRA-CSHDEDAGVTC
bWC1.1   983  GSAHFGAGSGPIWLDDLNCTGKESHVWRCPSRGWGRHD-CRHKEDAGVIC
Speract   94  RRAYFGAHVTTFWVYKMNCLGNETRLEDCYHRPYGRPWLCNAQWAAGVEC
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FIG. 3. Sequence alignment comparing the SRCR domain of CyCAP with other SRCR-containing proteins. The aligned sequences and their respective amino acid identity with CyCAP are as follows: bovine macrophage scavenger receptor type I (bMSR), 54% (32); bovine WC1.1 protein domain 9 (bWC1.1), 53% (36); sea urchin speract receptor domain 1, 36% (35). The six precisely spaced cysteines are outlined, and amino acids identical in all four proteins are shown in boldface type. mCyCAP, murine CyCAP.

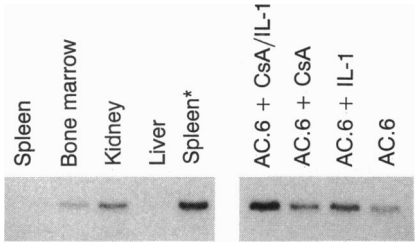

FIG. 4. RNA analysis of CyCAP gene expression. Northern analysis of CyCAP expression in selected tissues shows that its expression profile matches that of Cyp-C. CyCAP expression is induced by addition of interleukin 1 (IL-1) in AC.6 cells. This expression is detectable in lymphoid tissues and the kidney but is not detectable in the liver. The sample marked Spleen* is splenic RNA from an animal undergoing hematopoietic reconstitution and is a source of early myeloid and erythroid cells. Twenty micrograms of total RNA was used in each lane.

stromal cells, is present in kidney and spleen, but is absent or weakly expressed in the liver.

Transfection of CyCAP cDNA into COS Cells Directs the Production of a 77-kDa Glycoprotein That Binds to Cyp-C. CyCAP cDNA was cloned into a modified pSR-α expression vector (29) and transfected into COS cells. Labeled proteins were prepared by addition of [^{35}S]cysteine and [^{35}S]methionine to cells 72 hr after transfection. Fig. 5 shows the results of Cyp-C–GST affinity isolation of labeled proteins; pSR-α–CyCAP-transfected cells, but not control transfectants, produce a 77-kDa protein that binds to Cyp-C–GST (Fig. 5*A*). The observed 77-kDa protein present in CyCAP-transfected cells will bind to Cyp-C in the absence (− CsA) but not the presence (+ CsA) of CsA (Fig. 5*B*). Treatment of recombinant CyCAP with endoglycosidase F/N-glycosidase F, which hydrolyzes N-glycans of the "high mannose" and "complex" types, shifts the molecular mass to 64 kDa in agreement with the predicted molecular mass (Fig. 5*C*).

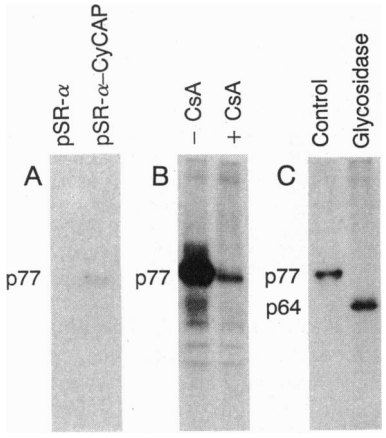

FIG. 5. (*A*) Demonstration of production of recombinant CyCAP in transfected COS cells. Metabolically labeled cell lysate protein (5×10^6 cpm) was used in each lane. COS cells transfected with vector alone do not express a protein that binds to Cyp-C (pSR-α), whereas cells transfected with pSR-α–CyCAP plasmid express a 77-kDa protein that binds to Cyp-C (pSR-α–CyCAP). (*B*) Demonstration of the CsA sensitivity of binding of recombinant CyCAP to Cyp-C. With 5×10^6 cpm of metabolically labeled protein from pSR-α–CyCAP-transfected cells for each affinity reaction, recombinant CyCAP will bind to Cyp-C in the absence (− CsA), but not in the presence (+ CsA), of CsA. These results are in agreement with results obtained by using native CyCAP purified from AC.6 cells. (*C*) Demonstration that CyCAP is a glycoprotein. In the first lane (control, buffer added), recombinant CyCAP produced in COS cells has an apparent molecular mass of ≈77 kDa. When CyCAP is digested with endoglycosidase F/N-glycosidase F (glycosidase), its molecular mass shifts to ≈64 kDa, in agreement with the mass predicted from the amino acid sequence.

DISCUSSION

We report here the purification of and corresponding cDNA clone for proteins demonstrated to have a high affinity for a cyclophilin in the absence of the drug CsA. The cDNA encodes a protein with a predicted molecular mass of 64 kDa and contains coding information derived from the purified 77-, 60-, and 37-kDa proteins. We propose that this protein be provisionally referred to as CyCAP until its function is known. Transfection studies using COS cells reveal that the CyCAP cDNA directs the production of a glycoprotein of 77 kDa, in agreement with initial results using AC.6 cells. Treatment of the protein with glycosidase reveals a core polypeptide of 64 kDa, in agreement with the predicted molecular mass based on sequence. Thus CyCAP, a glycoprotein with a N-terminal signal sequence, is probably a secreted molecule.

CyCAP belongs to a family of transmembrane and secreted proteins containing SRCR domains (31). Thus far, no function has been ascribed to the SRCR domain, although it appears to be dispensable for the function of at least one of the proteins in which it occurs, the macrophage scavenger receptor, as one form of the scavenger receptor lacks the SRCR domain but is still capable of ligand binding and endocytosis (34). However, the extracellular domain of the speract receptor is 91% SRCR domains, and in this case the domains are presumed to mediate ligand binding (35).

Previous studies have demonstrated that cyclophilins and FK506-binding proteins have the potential to intersect and inhibit an intracellular signaling pathway in the presence of their cognate drugs CsA and FK506. With the identification of calcineurin as the common target through which immunophilin–drug complexes block signal transduction in susceptible cells, the virtually identical bioactivities of CsA and FK506 can be understood. There remains, however, a central question: What is the role of the immunophilins in the absence of these exogenous ligands? The results shown here in combination with data on FK506-binding protein 59 (37, 38), the *Drosophila melanogaster* cyclophilin homologue *ninaA* (8, 12, 13), and recent work on Cyp-A (39) suggest that immunophilins as a family are likely to have pleiotropic effects, whereas the function of a particular immunophilin may be highly specialized.

An analogy can be drawn between the Cyp-C–CyCAP interaction and the *ninaA*–Rh 1 rhodopsin interaction seen in *D. melanogaster*, where *ninaA* is a cyclophilin homologue and Rh 1 rhodopsin is a presumed target of its actions. Although a physical association has yet to be demonstrated between *ninaA* and Rh 1 rhodopsin, in cells bearing *ninaA* mutations a massive amount of Rh 1 rhodopsin accumulates in the endoplasmic reticulum. It has been proposed that *ninaA* facilitates the cis/trans isomerization of a particular proline residue in its target rhodopsin molecule, thus allowing for proper processing and transport of the molecule (8, 12). CyCAP and Cyp-C interact with high affinity in solution, but we have yet to elucidate the functional significance of this interaction. Both proteins are likely to be in the lumen of the endoplasmic reticulum; CyCAP is a glycoprotein with a signal sequence, and Cyp-C can be found on the surface of viable cells (M.T., unpublished work). Perhaps Cyp-C is essential for the proper folding and processing of CyCAP or CyCAP may itself be part of a protein processing system, working in concert with Cyp-C on as-yet-unidentified target molecules. Mutagenesis studies of Cyp-C, similar to *ninaA*, may help to resolve these questions (40), as will expression studies using CyCAP in the presence or absence of a functional Cyp-C protein.

Another possibility is that CyCAP acts as a molecular bridge between Cyp-C and a signaling molecule. Interest-

ingly, the immunophilin FK506-binding protein 59 provides precedent for this type of interaction, as it has been shown to complex with the unliganded glucocorticoid receptor via heat shock protein 90 (37, 38). It is unlikely that a CyCAP–Cyp-C complex interacts functionally with calcineurin, as initially proposed (6, 14), as calcineurin A and B chains are cytoplasmic, and neither Cyp-C nor CyCAP appear localized to the cytoplasm (M.T., unpublished work). Also Cyp-C transfected into Jurkat cells fails to mediate CsA-induced calcineurin complexes and signal transduction inhibition unless its N-terminal sequence is deleted. (R. Bram and G. Crabtree, personal communication).

The interaction of CyCAP with Cyp-C is inhibitable by CsA. On the basis of the results of structural studies of Cyp-A, cyclophilins are not thought to undergo a significant conformational change upon binding of CsA (41, 42). Structure determinations also demonstrate that CsA and peptide substrates bind to the same pocket of Cyp-A (41–43). Therefore, the elution of CyCAP from Cyp-C by saturating amounts of CsA is likely to represent competitive binding. We propose that CyCAP be considered an endogenous CsA-like molecule, although a macromolecule that is in the secretory pathway. It will be of interest to identify the putative CsA-mimetic structure presented by CyCAP: this structure may prove useful for the design of peptide compounds with CsA-like properties. A potential problem with this appraisal is that one might expect Cyp-A and Cyp-B to bind CyCAP via their CsA-binding pocket, yet we have been unable to purify CyCAP by using analogous Cyp-A or Cyp-B affinity columns. Thus, despite the high degree of sequence conservation in the CsA-binding pocket between Cyp-A, Cyp-B, and Cyp-C, subtle differences are evident. These differences could be exploited in the development of compounds specific for particular cyclophilins and could prove useful when a better understanding of which cyclophilins mediate the immunosuppressive versus the toxic effects of CsA is available. Because CsA can induce nephrotoxicity as well as immunosuppression, the coexpression of Cyp-C and CyCAP mRNAs in the kidney could be a starting point for such investigations.

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