A Novel Cyclophilin from Parasitic and Free-living Nematodes with a Unique Substrate- and Drug-binding Domain*

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A highly diversified member of the cyclophilin family of peptidyl-prolyl cis-trans isomerases has been isolated from the human parasite Onchocerca volvulus (OvCYP-16). This 25-kDa cyclophilin shares 43–46% similarity to other filarial cyclophilins but does not belong to any of the groups previously defined in invertebrates or vertebrates. A homolog was also isolated from Caenorhabditis elegans (CeCYP-16). Both recombinant O. volvulus and C. elegans cyclophilins were found to possess an enzyme activity with similar substrate preference and insensitivity to cyclosporin A. They represent novel cyclophilins with important differences in the composition of the drug-binding site in particular, namely, a Glu124 (C. elegans) or Asp129 (O. volvulus) residue present in a critical position. Site-directed mutagenesis studies and kinetic characterization demonstrated that the single residue dictates the degree of binding to substrate and cyclosporin A. CeCYP-16::GFP-expressing lines were generated with expression in the anterior and posterior distal portions of the intestine, in all larval stages and adults. An exception was found in the dauer stage, where fluorescence was observed in both the cell bodies and processes of the ventral chord motor neurons but was absent from the intestine. These studies highlight the extensive diversification of cyclophilins in an important human parasite and a closely related model organism.

Cyclophilins belong to a large family of proteins that have been found in most organisms including parasites. It is thought that cyclophilins play an important role in protein folding because of their peptidyl-prolyl cis-trans isomerase (PPIase) activity, which can be measured in vivo (1, 2) and in vitro (3, 4). Most cyclophilins bind the immunosuppressive drug cyclosporin A (CsA), resulting in specific inhibition of their PPIase activity (5, 6). Therefore, CsA may interfere with the correct folding of proline-containing proteins that are the natural substrates for cyclophilins. It remains to be determined whether this is the mechanism by which CsA and its immunosuppressive derivatives exert lethal structural damage on a number of important parasites. For example, subimmunosuppressive levels of CsA cause gross herniation in the gut and blistering of the tegumental surface of Schistosoma mansoni (7). In the case of Litomosoides carinii microfilariae, the drug causes shrinkage of the parasite and stiffening of the surrounding sheath (8).

Most parasite cyclophilins published to date possess a high degree of similarity to human cyclophin A (CypA) (5), an 18-kDa cytoplasmic protein that is abundantly expressed in all mammalian tissues (9). Like human CypA, the PPIases described from S. mansoni (10–12), Toxoplasma gondii (13, 14), and Plasmodium falciparum (15) increase the rate of isomerization of a standard proline-containing peptide substrate (N-succinyl-Ala-Ala-Pro-Phe-nitroanilide) in vitro, and their PPIase activity is easily inhibited by nanomolar concentrations of CsA (9, 16–18). Thus far, only CypA homologs have been found in parasites, with the exception of the filarial worms. In addition to this highly conserved form, designated CYP-2 in filarial parasites, Brugia malayi, Onchocerca volvulus, and Dirofilaria immitis express two divergent cyclophilins that are more related to human nuclear-specific cyclophilin (CYP-3/4) and natural killer cell cyclophilin (CYP-1). These cyclophilins are considerably larger than human CypA, prefer other synthetic substrates, and display a reduced sensitivity to CsA (19–21).

We report here the cloning, expression, and characterization of a new class of cyclophilin from the important human parasite O. volvulus (OvCYP-16) and the model organism Caenorhabditis elegans (CeCYP-16). These cyclophilins are distinct from the other cyclophilins present in the data base from C. elegans (designated CeCYP-1 through CeCYP-15, and CeCYP-17) or O. volvulus (designated OcCYP-1, OcCYP-2, OcCYP-4, OcCYP-5, and OcCYP-10). The CYP-16 cyclophilins represent novel, highly diversified cyclophilins with respect to the composition of the drug-binding site and are particularly interesting because, unlike other parasite cyclophilins described thus far, they are apparently not found in mammals. We present molecular and biochemical studies on these new enzymes and use transgenic methodologies in C. elegans to analyze developmental and spatial expression of CeCYP-16 to gain insight into the potential natural substrate(s) for these enzymes.

EXPERIMENTAL PROCEDURES

Isolation of OcCYP-16 and CeCYP-16—All reagents, kits, and bacterial strains used in cloning, expression, and sequencing (described below) were obtained from New England Biolabs (Beverly, MA) and used as described by the manufacturer, unless otherwise specified. A partial cDNA clone (552 bp) encoding a putative cyclophilin was isolated from an O. volvulus L3 stage Lambda Uni-ZAP XR cDNA library kindly provided by Dr. Steven Williams. The library was screened by hybridization (22) with a 1300-bp genomic fragment of O. volvulus furin (a gift from Dr. Jingmin Jin). The fragment was radio-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank® and EBI Data Bank with accession number(s) AF017738 and AF393636.

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§ The abbreviations used are: PPIase, peptidyl-prolyl cis-trans isomerase; CypA, human cyclophilin A; CsA, cyclosporin A; MBP, maltose-binding protein; GFP, green fluorescent protein.
labeled with [α-32P]dATP by oligonucleotide random priming using the NEBlot kit. Plaque lifts (23) were hybridized in 6× SSC at 60°C overnight with the 32P-labeled probe (1 × 106 cpm/ml hybridization solution). After hybridization, the plaque lifts were washed twice at room temperature and once at 60°C for 30 min each in 2× SSC containing 0.1% SDS. After plaque purification of nine clones, the Bluescript phagemids were excised from Lambda ZAP (Stratagene, La Jolla, CA). After sequencing, the phagemid clone with the smallest insert (552 bp) and fragment B was identified as a fragment of a putative O. volvulus cyclophilin (OvCYP-16) by using the National Center for Biotechnology Information BLAST program. This clone was isolated due to significant homology in a short stretch of sequence (39 nucleotides) encoding part of the catalytic domain of each enzyme (data not shown).

To obtain a full-length cDNA of OvCYP-16, two primers, 5′-gggtggaaccttctgaccc-3′ (antisense) and 5′-cccaagcttctattcaactttattgaagaccgc-3′ (sense), were designed using the sequence derived from the 5′ and 3′ ends of the partial cDNA. Two PCR products, designated fragment A (241 bp) and fragment C (322 bp), were obtained by performing thermal cycling on partial cDNA. Two PCR products, designated fragment A (241 bp) and fragment C (322 bp), were designed using the sequence derived from the 5′ position in the drug-binding site is shown.

To obtain a full-length cDNA of OvCYP-16, two primers, 5′-gaatttgaaccttctgaccc-3′ (antisense) and 5′-cccaagcttctattcaactttattgaagaccgc-3′ (sense), were designed using the sequence derived from the 5′ and 3′ ends of the partial cDNA. Two PCR products, designated fragment A (241 bp) and fragment C (322 bp), were obtained by performing thermal cycling on partial cDNA. Two PCR products, designated fragment A (241 bp) and fragment C (322 bp), were designed using the sequence derived from the 5′ position in the drug-binding site is shown.

The amino acid present (aspartic acid (D), glutamic acid (E), histidine (H), tryptophan (W), or tyrosine (Y)) in the critical position in the drug-binding site is shown.

Preparation and Purification of Recombinant O. volvulus CYP-16 and C. elegans CYP-16—Thermal cycling primers were designed to enable cloning of OvCYP-16 into plasmid pMAL-c2X to generate a fusion with maltose-binding protein (MBP). The forward primer corresponded to the open reading frame of OvCYP-16 and had the sequence 5′-atgagtaatcaatatatcaacgagccg-3′, generating a 5′ blunt end. The reverse primer, 5′-cccaagcttctattcaactttattgaagaccgc-3′, corresponded to the 3′ end of the gene including a downstream termination codon and a HindIII recognition site. 50–μl PCR reactions were carried out using 0.1 ng of the pUC19-OvCYP-16 construct as template, 2 units of Vent DNA polymerase, 5 μl of 10× thermal polymerase buffer, 5 mm MgSO4, 0.2 μM deoxynucleotide triphosphate, and 250 nm of each primer. The thermal cycling conditions used were 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 1 min. The reaction product was purified and digested with HindIII before ligation into pMAL-c2X digested with XmnI and HindIII. Plasmid DNA was isolated, and the insert was sequenced in both directions using the CircumVent thermal cycle dideoxy DNA sequencing kit. Production and purification of the MBP fusion protein were as described by the manufacturer.

The CDNA clone yk648d4 (GenBank accession number AV195981) was obtained from Dr. Yuji Kohara, and two thermal cycling primers were designed to subclone OvCYP-16 into pMAL-c2X for protein expression. The forward primer (5′-atgagtaatcaatatatcaacgagccg-3′) corresponded to the open reading frame of OvCYP-16 preceded by an ATG codon. The reverse primer (5′-cccaagcttctattcaactttattgaagaccgc-3′) corresponded to the 3′ end of the gene and included a downstream termination codon and a HindIII recognition site. PCR reactions (50 μl) were performed as described above using 3 μl of yk648d4 Lambda DNA stock as template. PCR products were purified and digested with HindIII before ligation into pMAL-c2X digested with XmnI and HindIII. The recombinant plasmid DNA was isolated, and the insert was sequenced in both directions to ensure authenticity. Production and purification of the MBP fusion protein were as described by the manufacturer.

Production of Active Site Mutants of Filarial Cyclophilin—Site-directed mutagenesis of the PPIase domain of the previously described B.
malayi BmCYP-1 (19) was accomplished by the method of Kunkel (25).

The histidine residue (132) of BmCYP-1 was substituted with aspartic acid using the following mutagenic primer:

5'-H11032-attactacaacacctgcgcca-gatctcaatatatccatgtggtatttgg-3'.

The bases encoding the mutated amino acid are underlined. Mutagenesis of BmCYP-1 was verified using the CircumVent thermal cycle dideoxy DNA sequencing kit. The protocol used for the production and purification of BmCYP-1 (H132D) was as described previously (26).

Preparation and Purification of BmCYP-1, BmCYP-2, and DiCYP-3—The various filarial PPIases were prepared and purified as described previously (19, 21, 27).

Sequencing Analysis—DNA sequences were analyzed using the Genetics Computer Group (Madison, WI) software. Pairwise identity comparisons of OvCYP-16 and CeCYP-16 to other cyclophilins were performed using the program GAP. Alignment of the derived amino acid sequence of the enzyme domains of OvCYP-16, CeCYP-16, and other Fig. 3. Alignment of the deduced amino acid sequences of various cyclophilins. The amino acid sequences of various cyclophilins are denoted as follows (GenBank™ accession numbers are indicated in parentheses): D. immitis, DiCYP-1 (U70884), DiCYP-2 (U47813), DiCYP-3 (AF000668); B. malayi, BmCYP-1 (L37292), BmCYP-2 (U47811), and BmCYP-4 (AJ000916); O. volvulus, OvCYP-1 (U70827), OvCYP-2 (U47812), OvCYP-4 (AJ000917), and OvCYP-16 (AF017738); human nuclear-specific cyclophilin, HuCYP60 (U37219); human cyclophilin A, HuCYP60 (X52851); human natural killer cell cyclophilin, HuCYPNK (L04288); and C. elegans, CeCYP-4 (U36187), CeCYP-7 (U27539), CeCYP-8 (U31078), and CeCYP-16 (AF393636). C-terminal asterisks indicate translational terminations. In the CYP-1 and CYP-3 sequences, the additional C-terminal residues of CYP-1 and the additional N- and C-terminal residues of CYP-3 are not shown. Dashes indicate residues identical to the corresponding residue in OvCYP-16. Dots denote gaps. The residues important in CsA binding (33) are indicated with a #.

Diversified Cyclophilin from O. volvulus and C. elegans 14927
Diversified Cyclophilin from O. volvulus and C. elegans

**TABLE I**

| Substrate specificity of nematode cyclophilins toward various peptide substrates |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Ala-Xaa-Pro-Phe         | BmCYP-1                  | BmCYP-2                  | DiCYP-3                  | OcCYP-16                  |
| Small                   |                          |                          |                          |                          |
| -Ala-                   | 130,000 ± 24,894         | 610,000 ± 97,045         | 130,000 ± 16,774         | 3400 ± 0                 |
| -Gly-                   | 11,360 ± 366             | 51,040 ± 1000            | 8571 ± 1353              | 1190 ± 269               |
| Hydrophobic             |                          |                          |                          |                          |
| -Val-                   | 116,700 ± 19,450         | 558,200 ± 7066           | 71,430 ± 2041            | 4365 ± 412               |
| -Leu-                   | 690,000 ± 25,555         | 590,000 ± 112,380        | 48,000 ± 980             | 2778 ± 214               |
| -Ile-                   | 258,700 ± 2781           | 733,700 ± 32,465         | 52,380 ± 12,698          | 5532 ± 102               |
| -Nle-                   | 195,600 ± 22,678         | 985,900 ± 21,343         | 140,500 ± 5282           | 3571 ± 73                |
| Aromatic                |                          |                          |                          |                          |
| -Phe-                   | 41,000 ± 837             | 265,800 ± 0              | 19,050 ± 572             | 1389 ± 0                 |
| -Trp-                   | 6300 ± 1890              | 160,000 ± 13,333         | 8600 ± 2457              | 198 ± 0                  |
| -Phe-                   | 9433 ± 304               | 41,400 ± 1119            | 3571 ± 123               | 1386 ± 320               |
| Acidic and amide        |                          |                          |                          |                          |
| -Glu-                   | 60,000 ± 5916            | 430,000 ± 4943           | 33,000 ± 3787            | 2100 ± 75                |
| -Gln-                   | 110,410 ± 7066           | 733,700 ± 106,720        | 64,290 ± 1786            | 1389 ± 347               |
| Basic                   |                          |                          |                          |                          |
| -His-                   | 1894 ± 358               | 85,100 ± 20,023          | 191 ± 29                 | 0                       |
| -Lys-                   | 34,700 ± 890             | 430,600 ± 47,844         | 47,620 ± 1642            | 794 ± 458                |

FIG. 4. Progress curves for the PPlase activity of O. volvulus CYP-16 and C. elegans CYP-16 using the synthetic substrate N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide. a, nonenzymatic thermal isomerization; b, 10 μM MBP alone; c, 10 μM recombinant O. volvulus CYP-16 fusion with MBP; d, 10 μM recombinant C. elegans CYP-16 fusion with MBP.

cyclophilins was made using the program PILEUP (gap weight = 3.0, gap length weight = 0.1). Phylogenetic tree analysis was performed using the Clustal method (24) with the PAM250 residue weight table in the Magalign program in DNA star.

**Determination of Peptidyl-prolyl cis-trans Isomerase Activity and CsA Inhibition Assays**—The PPlase activity of OcCYP-16 and CeCYP-16 fusion proteins was determined by measuring the cis-trans conversion of 13 available synthetic peptide substrates of the general structure N-succinyl-Ala-Xaa-Pro-Phe-p-nitroanilide (Bachem), where Xaa is any of the 12 amino acids listed in Table I. Reactions were performed at 10 °C and monitored at 0.3-s intervals at 400 nm using a Beckman DU 640 spectrophotometer. Pseudo-first-order rate kinetics were calculated using the following formula: \( k_{\text{obs}} = \left( k_{\text{cat}}/K_m \right) [E] \).

To determine inhibition of enzyme activity by CsA, \( k_{\text{obs}} \), recombinant enzyme (30 nM to 10 μM) was preincubated for 1 h at 4 °C with CsA (1 nM to 5 μM), and the assay was performed as described above. Data were fitted into the following equation: \( k_{\text{obs}} = k_{\text{obs}}^0 (1 + [\text{CsA}] / IC_{50}) \), where \( k_{\text{obs}}^0 \) is \( k_{\text{obs}} \) in the absence of CsA (19).

**Nematode Culture**—Wild-type C. elegans were obtained from the Caenorhabditis Genetics Center (St. Paul, MN). Worms were main-
(27), and OvCYP-4 (31) from O. volvulus. CeCYP-16 is a novel C. elegans cyclophilin and shares 35–56% similarity to the 17 documented cyclophilins in the C. elegans genome. It does not belong to any of the previously characterized groups defined by Page et al. (32). A data base search revealed an absence of mammalian homologs of the OvCYP-16 and CeCYP-16 cyclophilins (Figs. 2 and 3). In contrast, homologs of the filarial CYP-1, CYP-2, and CYP-3/4 proteins are present in humans (Figs. 2 and 3).

There are 13 residues that constitute the CsA-binding site of human cyclophilin A (33) (Fig. 3, #), and one of these residues (tryptophan 121) is essential for drug binding (34). In the OvCYP-16 and CeCYP-16 cyclophilins, 9 and 11, respectively, of the 13 residues are conserved. However, unlike any other cyclophilins described to date, the tryptophan residue is substituted with a Glu124 (C. elegans) or Asp124 (O. volvulus) amino acid (Fig. 3).

Characterization of PPIase Activity and Inhibition Studies Using CsA—The characteristics of recombinant OvCYP-16- and CeCYP-16-MBP fusion proteins were examined using 13 different synthetic peptides of the general structure N-succinyl-Ala-Xaa-cis-Pro-Phe-p-nitroanilide, where Xaa is any of the 12 amino acids listed in Table I. The tripeptide substrate Suc-Phe-Pro-Phe-pNA was also evaluated. Mutant BmCYP-1 (H132D) and the previously characterized filarial cyclophilins BmCYP-1, BmCYP-2, and DiCYP-3 were included for comparison as MBP fusion proteins. BmCYP-1, BmCYP-2, and DiCYP-3 are active PPIases, both as a MBP fusion protein and in a purified (minus MBP) form (19, 21, 27).

The catalytic efficiency ($k_{cat}/K_m$) of the substrates varied, and a distinct profile was obtained for each filarial and C. elegans cyclophilin (Table I). Both OvCYP-16 and CeCYP-16 proteins were found to possess a low level of PPIase activity that was only detectable using relatively large amounts of protein (namely, 9.7 μM OvCYP-16 and 4 μM CeCYP-16, respectively) in the presence of specific substrates (Fig. 4). The highest level of PPIase activity ($k_{cat}/K_m$) for OvCYP-16 and CeCYP-16 was 5.2 × 10^6 (Ala-Leu-Pro-Phe or Ala-Nle-Pro-Phe) and 2 × 10^6 (Ala-Val-Pro-Phe), respectively. The profile observed for the mutant PPIase (5 μM BmCYP-1, H132D) was more similar to OvCYP-16 and CeCYP-16 and was dramatically reduced (2.7 × 10^6 Ala-Leu-Pro-Phe) compared with wild-type (6.9 × 10^6) (Table I).

To determine the sensitivity of OvCYP-16, CeCYP-16, and mutant BmCYP-1 (H132D) PPIases to CsA, recombinant enzyme (10 μM) was preincubated with varying concentrations of CsA (10 nM to 5 μM) at 4°C for 1 h before the assays were performed as described above. The previously characterized filarial PPIases, BmCYP-1, BmCYP-2, and DiCYP-3, were included for comparison. Appropriate synthetic substrates were used in the assays, namely, N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide for OvCYP-16 and CeCYP-16 and N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for BmCYP-1, BmCYP-1 (H132D), BmCYP-2, and DiCYP-3, respectively.

Expression Pattern of CYP-16 in C. elegans—Transgenic lines IP102 and IP103 were obtained after coinjection of pPI10 and pRF-4 into the gonad of adult worms. Consistent GFP expression patterns were observed in these lines throughout the intestine, with particularly strong fluorescence in the anterior and posterior ends, in all the larval stages and adults (Fig. 6). An exception was found in the dauer stage, where fluorescence was observed in both the cell bodies and processes of the ventral chord motor neurons but was absent from the intestine (Fig. 6).
Cyclophilins appear to have undergone proliferation and extensive diversification in filarial worms. In contrast to other parasites that appear to only express homologs of human CypA, the filariae possess, in addition, highly distinctive cyclophilins. The three filarial PPIases (CYP-1, CYP-2, and CYP-3) analyzed in detail thus far differ in size and display unique substrate preferences and a range of sensitivity to inhibition with CsA (21, 27, 32). The other cyclophilins (designated CYP-4, CYP-5, and CYP-10) identified from O. volvulus as potential vaccine and drug target candidates after immunoscreening of cDNA libraries and expressed sequence tag anal-

FIG. 6. Expression pattern of cyp-16::GFP. A, cyp-16::GFP expression in the adult hermaphrodite is seen throughout the intestine, with particularly strong expression in the anterior and posterior ends. Similar expression is seen in all detectable larval stages except the dauer stage. B, cyp-16::GFP expression in the dauer larva is seen in neuron cell bodies and processes along the length of the body, consistent with identification as the ventral chord motor neurons. Expression in the gut is absent in this stage.
It is known from the extensive work done on mammalian cyclophilins that the various isoforms can vary in their expression pattern (41). To gain information on the function of filarial CYP-16, properties of its homolog were studied in the genetic model C. elegans. Developmental and spatial expression patterns of CeCYP-16 were determined using a GFP reporter system. It was found that GFP expression under the control of the CeCYP-16 promoter was strong in the distal portions of the intestine. This pattern was seen throughout development, with the exception of the dauer larva, in which expression was seen only in the ventral chord motor neurons. Therefore, CeCYP-16 would seem to be functioning in distinct cell types during regular development as compared with the dauer stage. During the dauer stage, the intestine is closed off to the environment, and the animal does not feed (42). Therefore, possibly a protein functioning in the intestine would no longer be needed during this stage, resulting in a loss of expression in the gut. Of greater interest is the apparent recruitment of this protein in completely unrelated cells during the dauer stage. Because dauer animals undergo less movement compared with other developmental stages, one might speculate that a dauer-specific function for CeCYP-16 in the motor neurons might be of an inhibitory nature. It would be interesting to determine whether a parallel expression pattern is seen in filarial parasites, where the dauer stage is represented by the infective stage larva that remains in the insect vector until transmitted to the mammalian host.

The expression pattern of CeCYP-16 resembles that of CeCYP-8, another cyclophilin with a homolog in filarial parasites (BmCYP-1). CeCYP-8 was found specifically in gut cells in various stages (39). However, this study did not include the dauer stage, so it remains to be seen whether there is complete overlap in the expression patterns of these two proteins. A completely different pattern was observed for CeCYP-4, a muscle-specific cyclophilin that has been shown to be essential for normal muscle development in early larvae. RNA interference experiments of the CeCYP-4 resulted in progeny with a lumpy appearance (31). Similar experiments performed using CeCYP-8 (39) or CeCYP-16 (data not shown) did not show any obvious phenotype, perhaps indicating that they perform the same function in the gut, although this is unlikely due to the distinct substrate preference profile observed for CeCYP-16 and BmCYP-1 (a homolog of CeCYP-8). It is also noteworthy that RNA interference is ineffective against neuronal expressed products (43), so other approaches would be necessary to address the importance of CeCYP-16 in this tissue.

Additional studies on both conserved and divergent cyclophilins in nematodes may give insight into the function of the various PPIases and reveal potential drug targets for antifilarial chemotherapy.

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