Molecular Characterization of a Cyclosporin A-Insensitive Cyclophilin from the Parasitic Nematode *Brugia malayi*[†,‡]

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ABSTRACT: The cyclophilins are a family of proteins that exhibit peptidyl-prolyl *cis*−*trans* isomerase (PPIase, EC 5.2.1.8) activity and bind the immunosuppressive agent cyclosporin A (CsA) to varying degrees. We have isolated a cDNA clone encoding a novel cyclophilin from the human filarial parasite *Brugia malayi*. This gene possesses an N-terminal domain homologous to cyclophilins from diverse phyla (49−60% amino acid sequence identity) and a hydrophilic C-terminal domain. The cyclophilin domain was overexpressed in *Escherichia coli* and found to possess peptidyl-prolyl *cis*−*trans* isomerase (PPIase) activity, with a k_{cat}/K_m value of 7.9×10^6 M^{-1} s^{-1}. A histidine residue in lieu of tryptophan in the highly conserved CsA-binding site suggests that *B. malayi* cyclophilin is more closely related to the cyclophilin-like proteins described recently from natural killer (NK) cells, plants, and the 40 kDa cyclophilins from mammals. In accordance with the histidine-containing CsA-binding domain, the *B. malayi* enzyme was relatively insensitive to inhibition by CsA, since an IC_{50} value of 860 nM (compared to 19 nM for human cyclophilin A) was determined.

Brugia malayi is a filarial nematode parasite of humans with widespread distribution in tropical Asia. The parasite resides in the lymphatic system and causes acute fevers, adenolymphangitis, and lymphadenitis, which may be followed in later chronic stages of infection by elephantiasis. Collectively, the various species of filarial parasites are estimated to infect approximately 100 million people worldwide, and over 1 billion people live in areas where filariasis is common (World Health Organization, 1991).

The cyclophilins are a family of proteins that exhibit peptidyl-prolyl *cis*−*trans* isomerase (PPIase[1] or rotamase, EC 5.2.1.8) activity (Fischer et al., 1989) and bind the immunosuppressive agent cyclosporin A (CsA) to varying degrees. The PPIase activity of cyclophilins accelerates the *cis* to *trans* isomerization of Xaa−Pro bonds, and in most cases, CsA inhibits this enzymatic activity (Takahashi et al., 1989). Cyclophilins are thought to play a critical role in protein folding since they have been shown to accelerate the refolding of several proteins *in vitro* (Lang et al., 1987; Fransson et al., 1992; Gething & Sambrook, 1992) and *in vivo* (Lodish & Kong, 1991; Steinmann et al., 1991).

CsA is a fungal cyclic undecapeptide that is used extensively for the treatment of autoimmune diseases and prevention of graft rejection. The molecular events involved in the immunosuppressive action of this drug have recently been determined. It is now known that CsA acts as a prodrug, and it is the CsA/cyclophilin complex that blocks signal transduction events in the T-cell, at a step between T-cell receptor stimulation and cytokine gene transcription. The target of this toxic complex is the protein serine phosphatase calcineurin, which is responsible for the dephosphorylation of important transcription factors (Schreiber & Crabtree, 1992).

CsA also possesses potent antiparasitic activity, effective against many protozoa and helminth species (Chappell & Wastling, 1992). The mode of action of CsA against these organisms is not known; however, CsA-binding cyclophilins have been found in the CsA-sensitive parasites *Toxoplasma gondii* (High et al., 1994), *Schistosoma mansoni* (Koletsky et al., 1986), and *Plasmodium falciparum* (Bell et al., 1994).

In this study, we describe the cloning and overexpression of a novel cyclophilin-like protein from *B. malayi*. This protein contains two domains: an N-terminal cyclophilin domain and a charged, hydrophilic C-terminal domain. The unique residues in the CsA-binding site and the presence of a C-terminal extension indicate that the *Brugia* cyclophilin is unlike the parasite cyclophilins described to date and is most closely related to the larger cyclophilin-like proteins (Anderson et al., 1993; Kieffer et al., 1993). Recombinant *B. malayi* cyclophilin possesses PPIase activity, which displays a reduced sensitivity to CsA when compared to cyclophilins with totally conserved CsA-binding domains.

MATERIALS AND METHODS

Isolation of the B. malayi Cyclophilin Gene Bmcyp-1. In the process of screening a λgtll cDNA library made from adult male *B. malayi* parasites, the cyclophilin gene was isolated serendipitously. The cloned insert was amplified by PCR using λgtll universal primers and labeled with [α-^{32}P]-dATP by random priming. Approximately 600 000 phages were screened on duplicate filters prepared using the Benton−Davis plaque lift method (Benton & Davis, 1977). A single hybridizing plaque, cBmcyp-1, was identified on duplicate filters and plaque purified. cBmcyp-1 contained

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[‡] The nucleotide sequence data reported in this paper have been deposited in the GenBank database under accession number L37292.

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[1] Abbreviations: PPIase, peptidyl-prolyl *cis*−*trans* isomerase; CsA, cyclosporin A; MBP, maltose-binding protein.

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a 1784 bp insert and was found to be a partial clone. To obtain a full-length coding sequence, a genomic library was rescreened with a probe corresponding to the 3′ end of cBmcyp-1. This probe was prepared by *Hinc*II/*Eco*RI digestion on the phage DNA to generate a 1214 bp insert. Three copies of an overlapping genomic clone (gBmcyp-1) were obtained (see Figure 1A). gBmcyp-1 contained a 2065 bp insert and was sequenced. The coding region was further confirmed by sequencing an adult cDNA reverse-transcribed PCR product (RTBmcyp-1) generated with primers corresponding to the ends of the cBmcyp-1 and gBmcyp-1 clones. Poly(A) mRNA was obtained by using the Micro-Fast Track Kit (InVitrogen), and cDNA was synthesized by using a cDNA synthesis kit (Amersham).

Phage DNA Preparation, Subcloning, and Sequencing. cBmcyp-1 and gBmcyp-1 DNAs were purified from liquid phage preparations and subsequently concentrated on CsCl gradients (Sambrook et al., 1989). *Eco*RI digestion of cBmcyp-1 DNA produced two fragments, and both were subcloned independently into pUC19 and sequenced. Similar digestion of gBmcyp-1 produced one fragment, which was also subcloned and sequenced. The complete sequence of these clones was determined in both directions by using the pUC19 universal primers and primers designed specifically from the derived sequences. DNA sequences were aligned and compared by using the University of Wisconsin Genetics Computer Group software, and searches for homologies to the encoded protein sequence were performed using BLAST (Altschul et al., 1990) and GCG FASTA programs (Pearson & Lipman, 1988).

Genomic Southern Blot Analysis. Aliquots (10 μg/lane) of genomic DNA from *B. malayi* or *B. pahangi* were digested with *Hin*dIII, separated by electrophoresis, and transferred to nitro-cellulose (Sambrook et al., 1989). The filter was hybridized overnight with [α-^{32}P]dATP-labeled cBmcyp-1 at 37 or 22 °C in 30 mM Tris (pH 7.5), 10 mM EDTA, 50% formamide, 2% SDS, and 0.1 × SSC. After the membrane was washed in 0.1× SSC and 0.1% SDS at 55 or 22 °C, it was autoradiographed.

Preparation and Purification of Recombinant B. malayi Cyclophilin. Thermal cycling primers were designed to enable cloning of the cyclophilin domain of Bmcyp-1 into the plasmid pMAL-c2 (New England Biolabs) to generate a fusion protein with maltose-binding protein (MBP). The forward PCR primer corresponded to the open reading frame of Bmcyp-1, with the addition of an upstream *Bam*HI recognition site, and had the sequence 5′-ggggatccatgtcaaaaaaagatcgccg-3′ (Figure 1B, DNA sequence underlined). The reverse PCR primer used corresponded to the 3′ end of the enzyme domain (underlined), and a downstream termination codon and *Hin*dIII recognition site were included to produce the following primer sequence: 5′-cggaagcttcaaacaagttcaccacaattaagtat-3′ (Figure 1B, DNA sequence underlined). Plasmid DNA was isolated, and the insert was sequenced in both directions to ensure authenticity. The protocol used for the production and purification of the MBP fusion protein was as described by the manufacturer. In a typical experiment the yield of fusion protein was 20 mg/L. Cleavage of MBP from the fusion protein was performed overnight with 1% (w/v) factor Xa protease. Recombinant cyclophilin was purified to homogeneity by fast protein liquid chromatography (FPLC) using a Mono Q anion exchange resin (Pharmacia).

Peptidyl-Prolyl cis−trans Isomerase (PPIase) Activity and CsA Inhibition. The PPIase activity of *B. malayi* cyclophilin was determined by measuring the *cis−trans* conversion of the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Fischer et al., 1989), with a modification of the substrate solvent (Kofron et al., 1991). Reactions were performed at 9.5 °C and monitored at 0.3 s intervals at 400 nm using a Beckman DU 640 spectrophotometer. First-order rate kinetics were observed with a rate constant $k_{obs} = (k_{cat}/K_m)[E]$. To determine the inhibition of enzyme activity by CsA (Sandoz), recombinant enzyme (15 nM) was preincubated for 1 h at 4 °C with CsA (10 nM to 3 μM), and the assay was performed as before. Data were fitted to the following equation: $K_{obs} = K_{obs}^*/(1 + [CsA]/IC_{50})$, where K_{obs}^* is K_{obs} in the absence of CsA.

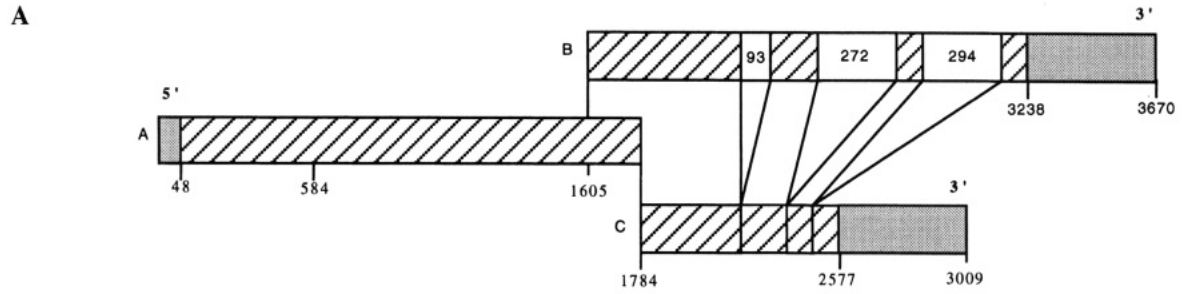
RESULTS

Cloning of B. malayi Cyclophilin. The partial cDNA clone (cBmcyp-1) encoding the *B. malayi* cyclophilin-like protein possesses a continuous open reading frame of 1784 bp, with a putative initiation codon at position 48 (Figure 1B). The complete 3′ end of this gene was obtained from an overlapping genomic clone (gBmcyp-1), whose coding sequence was confirmed by RTPCR sequencing (RTBmcyp-1, outlined in Figure 1A). The complete gene encodes an 843 aa protein, which is followed by a 429 bp noncoding region. This translated protein has a predicted molecular mass of approximately 93 000 Da with a p*I* of 11 and contains two distinct domains.

The 177 aa N-terminal domain is similar to cyclophilins from diverse species with sequence identities ranging from 49 to 60% (Figure 2). The *B. malayi* cyclophilin is most closely related to the cyclophilins from NK cells (Anderson et al., 1993), plants (Bartling et al., 1992), and cyclophilin-40 (Kieffer et al., 1993) based on the presence of an 8 amino acid insert (residues 51−58, Figure 2 underlined). The GK dipeptide of this insert is conserved in all of these cyclophilins, and five amino acids (GKPLH) are identical between *Brugia* and the cyclophilin-40 protein. A further unifying feature of this group of cyclophilins is the presence of a histidine residue in lieu of tryptophan in the CsA-binding site (position 132, Figure 2, indicated ψ). There are 13 residues that constitute the CsA-binding site of human cyclophilin A (Pfugl et al., 1993) (Figure 2, indicated #), 11 of which are conserved in *Brugia* cyclophilin. The tryptophan residue (Figure 2, human cyclophilin A, HA, position 121 ψ) has been determined to be essential for drug binding (Lui et al., 1990; Bossard et al., 1991). The other CsA-binding residue difference in the *Brugia* cyclophilin occurs at position 114 (Figure 2, alanine to lysine).

The C-terminal domains of the *B. malayi* (666 amino acids) and NK cell proteins are also conserved with 17% identity or 68% overall similarity in a 417 aa overlap (*B. malayi*, residues 427−843; human NK, residues 449−897). Like the NK cell cyclophilin, this domain is extremely hydrophilic, is positively charged, and contains many serine, lysine, and arginine residues.

Genomic Southern Blot Analysis. Southern blot analysis was performed by using the entire cBmcyp-1 cDNA as a probe. A single hybridizing band of approximately 6 kb was observed in both *B. malayi* and the closely related filarial species *B. pahangi* (Figure 3). This result was independent of the stringency employed (data not shown).



B

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   1  CGAAATAATGCTAATTTTCTTATTTAATCCTACTATTGTGACGGAAA#ATGTCAAAAAAAGATCGCCGCCGGGTATTTTTGGATGTAACAATTGATGGTA
 (1)                                                M  S  K  K  D  R  R  R  V  F  L  D  V  T  I  D  G  N
 100 ACCTTGCGGGTCGAATTGTGATGGAATTGTACAATGATATAGCACCACGGACGTGTAATAATTTCCTGATGCTTTGTACTGGTATGGCAGGTACCGGTA
(19)   L  A  G  R  I  V  M  E  L  Y  N  D  I  A  P  R  T  C  N  N  F  L  M  L  C  T  G  M  A  G  T  G  K
 199 AGATTAGTGGCAAACCTTTGCACTACAAAGGATCAACATTTCATCGTGTCATCAAAAATTTCATGATTCAGGGAGGTGATTTTACGAAAGGTGACGGTA
(52)   I  S  G  K  P  L  H  Y  K  G  S  T  F  H  R  V  I  K  N  F  M  I  Q  G  G  D  F  T  K  G  D  G  T
 298 CAGGTGGGGAATCAATTTATGGTGGTATGTTTGACGATGAGGAATTCGTTATGAAACATGATGAACCGTTTGTTGTGTCGATGGCGAACAAGGGACCTA
(85)   G  G  E  S  I  Y  G  G  M  F  D  D  E  E  F  V  M  K  H  D  E  P  F  V  V  S  M  A  N  K  G  P  N
 397 ATACGAATGGTTCACAGTTTTCATTACTACAACACCTGCGCCACATCTCAATAATATCCATGTGGTATTTGGTAAGGTTGTTTCTGGGCAGGAAGTTG
(118)  T  N  G  S  Q  F  F  I  T  T  T  P  A  P  H  L  N  N  I  H  V  V  F  G  K  V  V  S  G  Q  E  V  V
 496 TAACCAAAATCGAATATTTAAAAACTAATTCCAAGAATCGTCCACTAGCTGATGTTGTAATACTTAATTGTGGTGAACTTGTTCGACGAAAAAAACGTC
(151)  T  K  I  E  Y  L  K  T  N  S  K  N  R  P  L  A  D  V  V  I  L  N  C  G  E  L  V  R  R  K  K  R  Q
 595 AACATTCTTCTAGATCAAATGAATCAGTCAGTTCTTCTACATCAACTGAAAAAAGTCACAAAAAGACAAAAAAGACAAAAATGAAAGAAAAGAAGCGGA
(184)  H  S  S  R  S  N  E  S  V  S  S  S  T  S  T  E  K  S  H  K  K  T  K  K  T  K  M  K  E  K  K  R  K
 694 AAGAGAGTGATGAAGTGGAACAATTGGAAATTGGTACTGTTGTTCCGGAAGCAGAACTGCAGTTATCGAGCGTAAAAGCTGAAGATTTGCCTGATGAAC
(217)  E  S  D  E  V  E  Q  L  E  I  G  T  V  V  P  E  A  E  L  Q  L  S  S  V  K  A  E  D  L  P  D  E  P
 793 CAGATCACCAAAATAAATATCTTATGAGACGATCAAAAACGCCAGAAAATTCGAGGAAAGGAAAAAAAGAAAAGCAACGACAATCACCTCATCGCTTTT
(250)  D  H  Q  N  K  Y  L  M  R  R  S  K  T  P  E  N  S  R  K  G  K  K  E  K  Q  R  Q  S  P  H  R  F  S
 892 CGCGACGCGATATTGGTCATCGTTTGAATCGTATGCGGAGAACGCGAACCGGACATAAAATAAAGGGTCGTGGTGCACTTAGATTTCGAACTCCAGAGG
(283)  R  R  D  I  G  H  R  L  N  R  M  R  R  T  R  T  G  H  K  I  K  G  R  G  A  L  R  F  R  T  P  E  G
 991 GTAGTAGCGACCACGATGGGAGTCGTACTCCTCCCCATTGGAGGCGTGAACAGAATCGTGTAATAACACTTGATGAATTGCATCGTTTGCAAGAGAAAA
(316)  S  S  D  H  D  G  S  R  T  P  P  H  W  R  R  E  Q  N  R  V  I  T  L  D  E  L  H  R  L  Q  E  K  R
1090 GGAAAGCATATGAGCTTGAAGAACTTGAGAATCCCAAAAATGATGTCGTCGATAAAGCAAAAACTGGTATATTATTAAACACATCGGAGAAAATTGAAG
(349)  K  A  Y  E  L  E  E  L  E  N  P  K  N  D  V  V  D  K  A  K  T  G  I  L  L  N  T  S  E  K  I  E  D
1189 ACAAAGAGGAAAGGTATCGCGGTAAGTCTGAAAAGAAGGAAAATCGGCATGAGCGAAGTAGGCATACAACGCGACGGTCACCGGAGCATGTAACACGAC
(382)  K  E  E  R  Y  R  G  K  S  E  K  K  E  N  R  H  E  R  S  R  H  T  T  R  R  S  P  E  H  V  T  R  H
1288 ATTTTGTGAAGGAAAAAAATCGGCATAAAGTTGATGAGGTTGGGAACAGTGAAGATATGAAACAGACAAAAAGAGATCGACGAGGGCGAGCCGATGAAA
(415)  F  V  K  E  K  N  R  H  K  V  D  E  V  G  N  S  E  D  M  K  Q  T  K  R  D  R  R  G  R  A  D  E  K
1387 AAGAGAAAGTCGAAGTTAATGGTGAAAAAGCTGCTGCAATGGATGAGTTAAATCTGGATGAACCAACAGTAGAGGTTACATTGGACAGTGCCGAAGATA
(448)  E  K  V  E  V  N  G  E  K  A  A  A  M  D  E  L  N  L  D  E  P  T  V  E  V  T  L  D  S  A  E  D  I
1486 TAAGAGATAGTGATGACGAAGCCATTAGGATTCATTTATTGAAAGCAAAAAAAATGGCAGAAGAGAAAACGAAACAAGAAGCAAAGATGCTTGAAAAGA
(481)  R  D  S  D  D  E  A  I  R  I  H  L  L  K  A  K  K  M  A  E  E  K  T  K  Q  E  A  K  M  L  E  K  T
1585 CTGGTGATAAAGAAGGACGAGATCAAAAGACGATTTCTGAGGCGAAACAGAAGGACAGTGCTGAAAAAGATAGGCAGCATCGAGAGCATAAAAATGATG
(514)  G  D  K  E  G  R  D  Q  K  T  I  S  E  A  K  Q  K  D  S  A  E  K  D  R  Q  H  R  E  H  K  N  D  E
1684 AACTTGAAAAGCGAGCTATTGAGAAACAAGATAAAGATCAAATTGTAGAGAGAGATACAGGGAGTAAACAACGACGAAAAAGTGATAGCAAAGAACACA
(547)  L  E  K  R  A  I  E  K  Q  D  K  D  Q  I  V  E  R  D  T  G  S  K  Q  R  R  K  S  D  S  K  E  H  R
1783 GAGGAAAAACAGATCGAAAACATAGGAGCAAAAGCATTGAGGAAGATGGAAGACGAAGTACTAGTCGCGAAAAACTCGACGATTTGAAAAGAAAGGAAA
(580)  G  K  T  D  R  K  H  R  S  K  S  I  E  E  D  G  R  R  S  T  S  R  E  K  L  D  D  L  K  R  K  E  T
1882 CTTCAGGACAAAAAAGTCAAGCTGATAGTGAGCAGACTGTAGAAGCAAAAACAAATGTGGTCGATTCTAACAGCGATAATTCGAAGATGTCAGTAAATG
(613)  S  G  Q  K  S  Q  A  D  S  E  Q  T  V  E  A  K  T  N  V  V  D  S  N  S  D  N  S  K  M  S  V  N  G
1981 GAAAATTGAAGGAAGTTAGTTCAACTAATAAGGAGAATGAAGTTTCGGAACAGAAAGATTTAAAAGCGGAGTCGACAAAATCAGAAGAAATTAAGCAGC
(646)  K  L  K  E  V  S  S  T  N  K  E  N  E  V  S  E  Q  K  D  L  K  A  E  S  T  K  S  E  E  I  K  Q  Q
2080 AAGTAAACGAGGTTTCCAGAAAGCAAAAAGGTGGTGAAAAACCGAAAGAACATAAACGAAATGAGAGAAGTCGCAGTCGGAGACGCCGAAGCAGAAGTA
(679)  V  N  E  V  S  R  K  Q  K  G  G  E  K  P  K  E  H  K  R  N  E  R  S  R  S  R  R  R  R  S  R  S  N
2179 ATGGCCGTAGGCGACGAAGTTCGAGCCGTCGTTCAAGATCACGCGATCGCCGCCATAAATCACGATCTCGATCGAGAGGTTACGTGCGTCGGTTTGAAG
(712)  G  R  R  R  R  S  S  S  R  R  S  R  S  R  D  R  R  H  K  S  R  S  R  S  R  G  Y  V  R  R  F  E  G
2278 GATGGTCTCGCTCTCGCCGGCCTACTAGACGAGAACTGTACGATGAGCGTATGCGCCGGGAACGTGAACGACGTCGAAGCTTTGATAGGTATTCAGACA
(745)  W  S  R  S  R  R  P  T  R  R  E  L  Y  D  E  R  M  R  R  E  R  E  R  R  R  S  F  D  R  Y  S  D  R
2377 GAAGGCGTACAAGAAGTCGAAGTGCTCGACGGGACAGTGATCGGCATTCGAGACGTAGCCGAAAACGTTCACCATCTTCTTCAAGTAGTAGCAGTGAAA
(778)  R  R  T  R  S  R  S  A  R  R  D  S  D  R  H  S  R  R  S  R  K  R  S  P  S  S  S  S  S  S  S  E  S
2476 GTTCATCAAGCGATTCCCGAAGCACTGCAAGCAGCAGTGCATCATCAAAGCGTTCCAGCAGTTCTGATTCAAGCAGAAGTTCTCGGAGCAGAAGCTCGA
(811)  S  S  S  D  S  R  S  T  A  S  S  S  A  S  S  K  R  S  S  S  S  D  S  S  R  S  S  R  S  R  S  S  N
2575 ATTAGTTGATTTTATTTACTTTTCTTGTTATGCACTTATCGTATATTTCGCTGAAATTTTGATTGTTATATCCACCGAAAAAAATCTTAAATTTCCAAA
2674 AAATTTCTAAAGTGTGAGTTATCGTCGATGTGATATTGCACGTATGGTTGCCCAAAATCTAGTAAATTCAATTTTGTTTGAATGAGCGGGGATTGAATT
2773 GAGAGAGAGGGAGAAAGTGAGGGACAGAAAACTTTTTCAAATTGAGCTTCAATCTCTTAGAAAATTCTGAATGGTTTAAGCATATTTTCTAGGTTATTG
2872 CTTCTATTTGCACACTTGTTAACTCTATTTGTTTTACTGCTTTGAGTCTATATTTAAGTTTTGTCACGTAGCATTTCCCAAACATTTTCTTTACTGGCC
2971 GAATTATTCATTTGCACACATACACACTATTCTGCATTT  3009
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FIGURE 1: (A) Schematic diagram illustrating the isolation of the *Brugia* cyclophilin gene Bmcyp-1. (A) corresponds to the 1784 bp cDNA clone cBmcyp-1, and (B) is the 2065 bp genomic clone gBmcyp-1, which overlaps with the 3′ end of cBmcyp-1 by 179 bps. (C) represents the reverse-transcribed PCR product RTBmcyp-1 from adult cDNA used to confirm the coding sequence of gBmcyp-1. Sizes are in base pairs, and sizes of introns are indicated within intron boxes (not shaded/hatched). Shaded boxes represent noncoding regions and hatched boxes represent coding sequences. (B) Nucleotide and deduced amino acid sequence of the *Brugia* cyclophilin gene Bmcyp-1. Nucleotides and amino acids (in parentheses) are numbered on the left. The ATG initiation codon is indicated with #, and the TAG termination codon is underlined. The primer sequences used to subclone the enzyme domain for protein expression are underlined.

Production of Recombinant B. malayi Cyclophilin. The 177 amino acid cyclophilin domain was overexpressed in *E. coli* as a fusion protein with MBP (Figure 4). The fusion protein was affinity purified on amylose resin as a single product with a molecular mass of approximately 65 kDa (lane A). This preparation was cleaved to completion from MBP with the protease factor Xa (lane B), resulting in two protein bands, namely, MBP at 43 kDa and the recombinant enzyme domain at 22 kDa. The 22 kDa recombinant cyclophilin was subsequently purified from the contaminating MBP by anion exchange chromatography (lane C).

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Bm   MSKKDRRRVFLDVTIDGNLAGRIVMELYNDIAPRTCNNFLMLCTGMAGTGKISGKPLHYK  60
HNK  .GAQ..PQCHF.IE.NREPV...MFQ.FS..C.K..K...C..S.EK.L..TT..K.C..  114
H40  P.NPSNP...F..D.G.ERV....L..FA..V.K.AE..RA....EK.I.HTT.....F.  69
At       MAHC.F.M..G.QP....I...FP.-V.K.AE..RA....EK.I.P-...KMT.E  53
Tg   L.TMPNP...F.IS..KKP....EF..FA.VV.K.AE..RA....EK...R-.....Y..  233
HA     MVNPT..F.IAV..EPL..VSF..FA.KV.K.AE..RA.S..EK.F.--------..  49
Dm     MSTLP...F.M.A.NEPL.......RS.VV.K.AE..RA....EK.F.--------..  50
Sj   KQ.RNLP...F.IR.GNADR.......RS..V...AE..RA....DR.F.--------.H  63
Eg       GVKC.F.IS.G.KP.....FA.FD.-V.K.VE..RA....EK.F.--------..  46
Sc       MSQ.YF..EA..QPI..V.FK.....V.K.AE..RA....EK.F.--------.A  47

         #    ## #        #                                  ###
Bm   GSTFHRVIKNFMIQGGDFTKGDGTGGESIYGGMFDDEEFVMKHDEPFVVSMANKGPNTNG 120
HNK  .......V..........SE.N.K........Y.K..N.IL...RA.LL....R.KH... 176
H40  .CP...I..K........SNQN.........EK.E..N.HY...RAGLL....A.R.... 129
At   ..V.....PK..L......L.N.R.......AK.A..N.IH..TT.GLL....A..G... 113
Tg   .CP...I.PQ..C......RMN.........EK.A..N.SY..S...LL....A...... 293
HA   ..C...I.PG..C......RHN....K....EK.E..N.IL..TG.GIL....A...... 109
Dm   ..I.....P...C......NHN....K....NK.P..N.EL..TGSGIL....A.A.... 110
Sj   NCC.....PQ..C.....V.......K....RK....N.QLR.EGFG.L....S...... 123
Eg   ..K...I.PG..C......A.N....K....SK.E..N.NH..SK.MML....A.K.... 106
Sc   ..P.....PD..L......A.N....K.....K.P..N.KKH..R.GLL....A...... 107
                ψ
       # #       ##   #
Bm   SQFFITTTPAPHLNNIHVVFGKVVSGQEVVTKIEYLKTNSKNRPLADVVILNCGELV  177
HNK  .......K.....DGV.....L.I..F..IEQ..N...DAAS..Y...RVID..V.  230
H40  .......V.T...DGK.....Q.IK.IG.ARIL.NVEVKGE-K.AKLC..AE....  184
At   .......VAT...DGK........E.MD..R...ATQ.DRGDK..SE.K.AK..Q.*  169
Tg   .......V.C.W.DGK........A.....KMM.-AEGR.NGQ.KCA.E.SS..Q.   348
HA   .....C.AKTEW.DGK.......KE.MNI.EAM.RFGSRNG-KTSKKIT.AD..Q.E* 164
Dm   .....C.VKTAW.D.K.....E..E.LD..K...SYGSQ.G-KTSKKIIVA.S.S.*  165
Sj   .....C..KCDW.DGK.....R..D..N..K.M.SVGSK.G-KVKEP.T.SR....I* 178
Eg   .......AVTSW.DGK.....E.E..ED..KDM.AVGSS.G-KTSQE.L.TD..Q.*  156
Sc   .......V.C.W.DGK.....E..D.YDI.K.V.S.GSP.G-ATK.RI.VAKS...*  157
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FIGURE 2: Alignment of the deduced amino acid sequences of the *Brugia* N-terminal cyclophilin domain with other eukaryotic cyclophilins. Abbreviations: Bm, the amino acid sequence of the *Brugia* cyclophilin domain; HNK, human natural killer cell (GP L04288); H40, human cyclophilin-40 (GP L11667); At, *Arabidopsis thaliana* (GP X63616); Tg, *Toxoplasma gondii* 20 (GP U04634); HA, human cyclophilin A (GP X52851); Dm, *Drosophila melanogaster* (GP M62398); Sj, *Schistosoma japonicum* (GP M93420); Eg, *Echinococcus granulosus* (GP J04664); Sc, *Saccharomyces cerevisae* (GP X17505). Dots represent amino acids identical with the *Brugia* cyclophilin; dashes were introduced for maximum alignment and stars indicate termination codons. The 8 amino acid insert common to the cyclophilin-like proteins is underlined. The residues important in cyclosporin A binding are denoted with # (30), while ψ denotes the tryptophan histidine difference found in the CsA-binding domains of the cyclophilin-like proteins.

PPIase Activity of Recombinant B. malayi Cyclophilin and Insensitivity to CsA. The recombinant cyclophilin preparation was determined to have PPIase activity catalyzing the isomerization of the prolyl−peptide bond in the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. A representative progressive curve for this enzymatic activity is shown in Figure 5. The preparation was analyzed both as a fusion and cleaved from MBP. The MBP fusion was determined to have $k_{cat}/K_m = 3.5 \times 10^5\ M^{-1}\ s^{-1}$ in this assay. A 23-fold increase in activity was observed following the removal of MBP: $k_{cat}/K_m = 7.9 \times 10^6\ M^{-1}\ s^{-1}$. Inhibition of the PPIase activity was determined by using a range of CsA concentrations (10−3000 nM). A concentration of 860 nM was required to inhibit 50% of the *Brugia* PPIase activity (IC_{50}) (Figure 6).

DISCUSSION

We describe the cloning of a gene that codes for a cyclophilin-like protein from the parasitic nematode *B. malayi*. The N-terminal 177 amino acids of this protein share between 49 and 60% identity with other common cyclophilins, but differ significantly due to the presence of an 8 amino acid insert and a histidine residue in lieu of tryptophan in the drug-binding site. These features indicate that the *B. malayi* cyclophilin is more closely related to the cyclophilins of plants (Bartling et al., 1992), NK cells (Anderson et al., 1993), and the cyclophilin-40 protein (Kieffer et al., 1993). It has been speculated that the insert, common to this group of cyclophilins, may be responsible for a conserved function such as a binding or recognition site (Kieffer et al., 1993). An interesting intermediate between the common cyclophi-

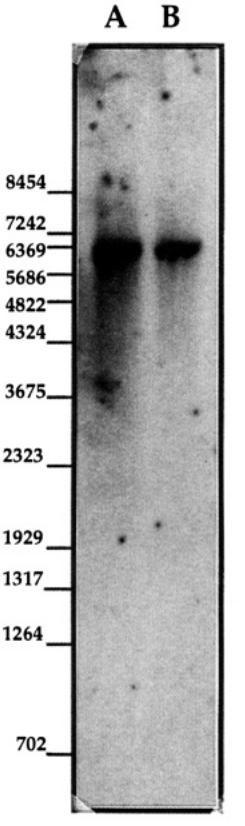

FIGURE 3: Southern blot analysis of *Brugia* genomic DNA using a cyclophilin probe. *Hin*dIII-digested DNA from *B. malayi* (lane A) and *B. pahangi* (lane B) was probed with an insert corresponding to the entire cBmcyp-1 cDNA at 37 °C overnight, followed by washing at 55 °C in 0.1× SSC/0.1% SDS.

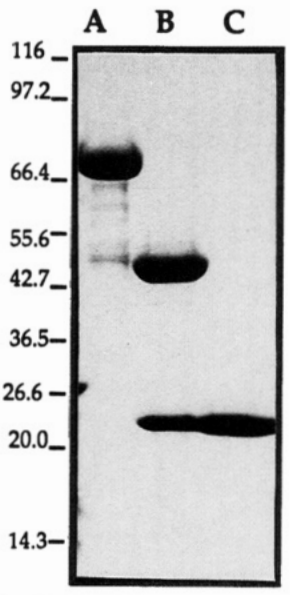

FIGURE 4: Analysis of recombinant *B. malayi* cyclophilin purified from *E. coli*. 10 μg of the protein samples was electrophoresed on a 4−20% gradient SDS−PAGE gel under reducing conditions and stained with Coomassie Brilliant Blue. Molecular mass standards are indicated in kilodaltons. Lane A: recombinant cyclophilin MBP fusion purified on an amylose resin. Lane B: material from lane A digested overnight at room temperature in the presence of 1% factor Xa. Lane C: material from lane B purified by anion exchange chromatography using the Mono Q column.

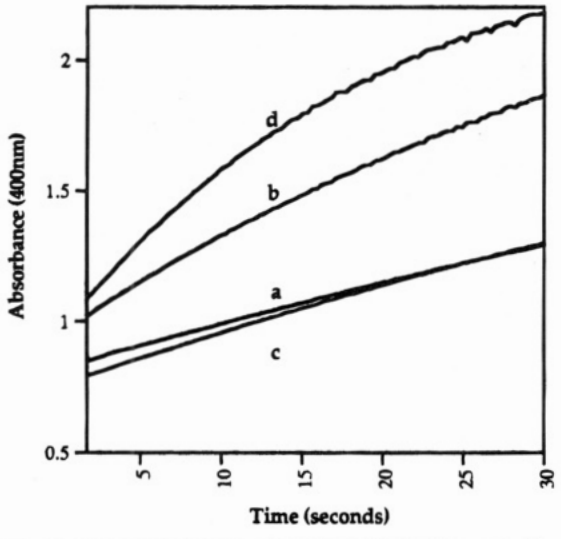

FIGURE 5: Typical progress curves for the PPIase activity: (a) nonenzymatic thermal isomerization; (b) 15 nM recombinant *B. malayi* PPIase cleaved from MBP; (c) 30 nM MBP alone; (d) 30 nM recombinant *B. malayi* PPIase cleaved from MBP.

lins and the cyclophilin-like proteins exists in *T. gondii* (High et al., 1994), whose cyclophilins contain both the insert and the conserved tryptophan in the drug-binding site. The C-terminal domains of the *B. malayi* and NK cell proteins are also conserved, with regions bearing significant homology to RNA/DNA-binding proteins (Zahler et al., 1992; Anderson et al., 1993).

Functional analysis of *E. coli*-expressed *B. malayi* cyclophilin demonstrated that it has potent PPIase activity, having a k_{cat}/K_m value of 7.9×10^6 M^{-1} s^{-1} at 9.5 °C in a purified form. This activity is relatively high for a recombinant cyclophilin, being similar to that reported for native human cyclophilin A ($k_{cat}/K_m = 1 \times 10^7$ M^{-1} s^{-1}) (Liu & Walsh, 1990). The lower k_{cat}/K_m value observed with the MBP fusion protein is similar to that determined for the NK cyclophilin−GST fusion protein: $k_{cat}/K_m = 7 \times 10^5$ M^{-1} s^{-1} (Rinfret et al., 1994). Surprisingly, expression of an MBP−enzyme domain construct with only 176 amino acids of the N-terminal cyclophilin domain (minus the valine residue; Figure 2, position 177) resulted in a protein that was completely inactive (data not shown).

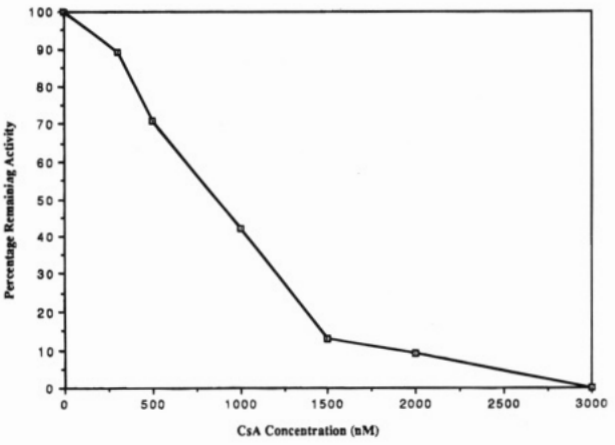

FIGURE 6: Typical plot of CsA inhibition of recombinant PPIase.

Since CsA is a known potent inhibitor of the PPIase activity of native human cyclophilin A (Lui et al., 1990), the effect of CsA on the PPIase activity of *B. malayi* cyclophilin was examined. An IC_{50} value of 860 nM was determined, indicating that compared to human cyclophilin A, with a reported IC_{50} value of 19 nM (Lui et al., 1990), the *B. malayi* PPIase displays a reduced (40-fold) sensitivity to the drug. Similarly high IC_{50} values of 760 and 300 nM have been described for the closely related cyclophilin-like proteins from NK cells (Rinfret et al., 1994) and the cyclophilin-40 protein (Kieffer et al., 1993), respectively. The tryptophan/histidine difference in the drug-binding site of these cyclophilins may be largely responsible for the greater IC_{50} values observed. It has been suggested that the reduced sensitivity to CsA may reflect an altered specificity for the putative natural ligands of these isomerases (Kieffer et al., 1992).

The ubiquitous nature of cyclophilins suggests that they play a fundamental role in development and/or metabolism. Vertebrate PPIase has been shown to accelerate the *in vitro* folding of type III collagens (Bachinger, 1987), and the *in vitro* folding of procollagen I is slowed significantly by low levels of CsA (Steinmann et al., 1991). Nevertheless, there is only one cyclophilin for which the natural physiological substrate has been determined. In *Drosophila*, the NinaA gene encodes a cyclophilin that is involved in the proper folding of rhodopsin Rh-1, and mutations in this gene result in impaired vision (Stamnes et al., 1991).

The relatively broad spectrum, antiparasitic effects of CsA are well documented (Chappell & Wastling, 1992). However, the mode of action of CsA against these organisms is not known, although it is likely to involve inhibition of PPIase activity or interruption of an essential signal transduction pathway. Interestingly, CsA-binding cyclophilins have been found in *Echinococcus granulosus* (Lightowlers et al., 1989), *Schistosoma japonicum* (Argaet & Mitchell, 1992), and the CsA-sensitive parasites *T. gondii* (High et al., 1994), *S. mansoni* (Koletsky et al., 1986), and *P.*

falciparum (Bell et al, 1994). As in the mammalian (Walsh et al., 1992) and yeast (Heitman et al., 1993) systems, it is now clear that multiple forms of this protein exist in parasites. *T. gondii* possesses two CsA-binding proteins, of 18.5 and 20 kDa, which are the products of different genes (High et al., 1994). The *B. malayi* protein represents a form that is unlike the other parasite cyclophilins described to date. It has an extended C-terminal non-cyclophilin domain and more closely resembles the NK cell cyclophilin. In addition, as would be predicted from the composition of its drug-binding domain, the *B. malayi* cyclophilin is relatively insensitive to inhibition by CsA, and we found that *B. malayi* parasites are nonsusceptible to relatively high levels of CsA *in vivo* (data not shown). Therefore, in the absence of differential drug uptake, differences in the composition of the drug-binding domains of parasite cyclophilins may explain the reported stage- and/or species-specific antiparasitic effects of CsA (Chappell & Wastling, 1992). Further studies on parasite cyclophilins are needed to determine the extent of variation in the drug-binding domains of these proteins, and studies on *B. malayi* cyclophilin in particular may lead to the identification of compounds capable of inhibiting this class of PPIases. Recently, non-immunosuppressive analogs of CsA were identified that possess potent activity against *P. falciparum* (Bell et al., 1994) and HIV virions (Thali et al., 1994). These findings suggest that distinct mechanisms may be involved in the antiparasitic/viral and immunosuppressive actions of CsA and open up the possibility of developing CsA derivatives for the treatment of important infectious diseases.

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