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Cloning and characterization of *Giardia intestinalis* cyclophilin

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Abstract: The cyclophilins (Cyps) are family members of proteins that exhibit peptidyl-prolyl cis-trans isomerase (PPIase, EC 5.2.1.8) activity and bind the immunosuppressive agent cyclosprin A (CsA) in varying degrees. During the process of random sequencing of a cDNA library made from *Giardia intestinalis* WB strain, the cyclophilin gene (*gicyp1*) was isolated. An open reading frame of *gicyp1* gene was 576 nucleotides, which corresponded to a translation product of 176 amino acids (Gicyp1). The identity with other Cyps was about 58-71%. The 13 residues that constituted the CsA binding site of human cyclophilin were also detected in the amino acid sequence of Gicyp1, including tryptophan residue essential for the drug binding. The single copy of the *gicyp1* gene was detected in the *G. intestinalis* chromosome by southern hybridization analysis. Recombinant Gicyp1 protein clearly accelerated the rate of *cis* → *trans* isomerization of the peptide substrate and the catalysis was completely inhibited by the addition of 0.5 μM CsA.
Key words: *Giardia intestinalis*, cyclophilins, cyclosporin, peptidylprolyl isomerase

INTRODUCTION

Giardia is a diplomonad protozoan parasite that causes intestinal infection in mammals, birds, reptiles, and amphibians. It is an important cause of waterborne and restaurant associated outbreaks of diarrhea, travelers' diarrhea and diarrhea in child care facilities (Adam, 2001). Because of absence of mitochondria and peroxisome organells present even in other protist and 16S ribosomal RNA sequence to indicate their ancient lineage (Sogin et al., 1993), *Giardia* is thought to be an important organism in evolutionary biology.

Cyclophilin (Cyp) proteins were first characterized in human and bovine spleen, based on their high-affinity binding to the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984). CsA was also shown to possess unexpected antiparasitic activities against schistosomes, plasmodia, cestodes, and nematodes (Chappell and Cha, 1988). Cyp proteins were found to have peptidylproryl *cis-trans* isomerase (PPIase) activity, thus catalysing the *cis-trans* isomerization of proline imidic bonds in peptides (Fischer et al., 1989).

Up to now, several cDNAs encoding Cyp proteins have been described. Cyp A, the homolog of the bovine protein, is cytosolic (Haendler et al., 1987), and Cyp B is found either in secretions or in the endoplasmic reticulum, possibly associated with calciosomes, after cleavage of signal peptide (Spik et al., 1991). Whereas Cyp C (less abundant form) is localized in the endoplasmic reticulum (Schneider et al., 1994), and Cyp D is probably mitochondrial (Bergsma, 1991) and Cyp-40 is found in association with the molecular chaperone Hsp90-steroid receptor complex (Duina et al., 1998).

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The sequence conservation, wide tissue distribution, and organelle targeting of the cyclophilin variants suggest that they possess important functions in many cell compartments. This view is supported by the discovery of cyclophilin homologs in prokariotic cells (Liu & Walsh, 1990) and various plants (Gasser et al., 1990).

The diverse effects of CsA reported in several parasitic infections have evoked an interest for Cyps from parasitic species. The CsA exerts an antimalarial activity in vivo and in vitro, and the inhibitory effect on the progress of lesions by *Leishimania* species is due to suppression of host T-lymphocyte activities (Solbach et al., 1986). McCabe et al. (1986) also reported that CsA has antiprotozoal and immunomodulatory activities in *Toxoplasma* infection. Recently, a Cyp protein of *Entamoeba histolytica* was reported, and it was demonstrated that the trophozoites were susceptible to the treatment with CsA.

Although there are numerous studies on Cyp and CsA action on parasites, no information is available as for *G. intestinalis*. In the present study, we describe isolation, expression and characterization of a gene coding for *Giardia* Cyp and CsA action on *Giardia* trophozoites.

MATERIALS AND METHODS

Giardia culture

Giardia intestinalis WB strain (ATCC 30957) was obtained from American Type Culture Collection and maintained in Keister's modified TYI-S-33 medium, supplemented with 10% fetal bovine serum at 37°C.

Identification and characterization of cDNA clone, *gicyp1*

In the process of EST's (express sequence tags) construction from a λZAP II cDNA library which was made from trophozoites of *G. intestinalis*, the cyclophilin gene was serendipitously isolated. The cloned insert, *gicyp1*, contained 536 bp and was found to be a partial clone. Sequence analysis and homology comparisons were performed using the program of the Genbank blast-search.

5′ RACE (rapid amplification of cDNA end) of *gicyp1*

To obtain a full-length coding sequence of *gicyp1*, 5′ RACE PCR was carried out by using Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA USA). PCR product was cloned into PGM-T vector (Promega, Madison, WI, USA) and sequenced using T7 sequencing kit (Amersham Biosciences, Piscataway, NJ, USA).

Hybridizations

The DNA probe was prepared from *gicyp1* cDNA clone. For Southern and northern blot analyses, cDNA probe with specific activity of $2\text{-}5 \times 10^8$ cpm/μg was made by Random labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) with ^{32}P (3000 Ci/mmol). Aliquots (10 μg/lane) of genomic DNA from *G. intestinalis* were digested with *Pst* I, *Eco* RI and *Hind* III (Roche). Northern blot analysis was carried out using 10 μg of total RNA prepared from trophozoite. Nucleic acids were separated by electrophoresis and transferred to nitro-cellulose membrane (BioRad, Hercules, USA). The pre-hybridized membranes were incubated for 1hr at 68°C with 2×10^6 cpm of nick-translated probes in Express hybridization solution (Clontech).

Construction of Gicyp1 expression vectors in *Escherichia coli*

Recombinant cyclophilin was expressed in *E. coli* using pGEX4T-1 (Smith and Johnson 1988). A *gicyp1* insert was obtained by complete digestion of *gicyp1* clone with *Eco* RI/*Xho* I (Posco, Pohang, Korea). This fragment was ligated into pGEX 4T-1 (Amersham Biosciences) that was digested with *Eco* RI/*Xho* I, and transformed into *E. coli* XL1-Blue. By Wisard mini prep (Promega), plasmid DNA was extracted from the ampicillin - containing overnight culture. The plasmid was digested with *Eco* RI/*Xho* I to release a cDNA insert (original insert size, 0.5 kbp) and retransformed into *E. coli* BL21. The overnight culture (200 ml) diluted 1:10 in fresh Luria Bertani media supplemented with ampicillin (50 μg/ml) was grown for 1 hr at 37°C before the addition of 0.3 mM IPTG. Following 3 hr of growth, cells were pelleted, resuspended in lysis buffer (containing 20 mM Tris pH 7.4,

500 mM NaCl, 10% glycerol, 1% NP-40), and sonicated four times at 15-s intervals. The supernatant was filtered with a 0.45-μm filter and passed over a glutathione-4B Sepharose column (Amersham Biosciences). The column was washed with phosphate-buffered saline (PBS) and eluted with 5 mM reduced-glutathione (Sigma, St. Louis. MI, USA) in 50 mM Tris-HCl (pH 8.0). After the removal of glutathione by filtration, the product was cleaved with human thrombin (Sigma) (25°C, 12 h) as described by Smith and Johnson (1988). The fusion protein, Gicyp1, was removed by passage over a glutathione-Sepharose column and the recombinant protein was analyzed by SDS-polyacrylamide gel electrophoresis.

Assay for PPIase enzyme activity of Gicyp1

PPIase activity of Gicyp1 was assayed in a Shimadzu UV-1201 spectrophotometer (Shimadzu, Japan), essentially as described (Fischer et al., 1989), with slight modification to measures the *cis* to *trans* isomerization of alanine proline peptide bond in the synthetic peptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma). Test peptide (2.1 mM) *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was dissolved in methanol (Standaert et al., 1990), and α-chymotrypsin (2 mg/ml in 100 mM Tris-HCl, pH 8.0) (Sigma) was added following preincubation of PPIase with the test peptide at 0°C. The reaction mixture (1 ml) containing the test peptide (63 μM), chymotrypsin (0.06 mg/ml), and varying amounts of Gicyp1 was placed into a spectrophotometer cell at 12°C. The first reading was taken at 5 sec to account for the dead time in the mixing of the sample, and readings were taken at 10-sec intervals. All reactions were performed in triplicate. For inhibition assay of CsA on PPIase activity of recombinant Gicyp1 protein, 0.5 μM CsA was added to the above PPIase activity test.

RESULTS

Cloning of *G. intestinalis* cyclophilin gene

The partial cDNA clone Gi054 encoding the *G. intestinalis* cyclophilin-like protein lacked a putative initiation codon ATG triplet. The 3′ end of this clone had TAA stop codon and a putative polyadenylation signal AGTAAA (Fig. 1). To obtain the complete 5′ end of this gene, 5′ RACE PCR amplification was performed on mRNA prepared from trophozoites using reverse primers constructed around the 5′ region of the *gicyp1* sequence. Sequencing of an amplification product revealed a 174 bp extension at the 5′ end of this clone (Fig. 2). An ORF (open reading frame) of *gicyp1* gene was 576 nucleotides, which corresponds to a translated product of 176 amino acids. The upstream sequence was A + T rich as has been reported for other giardial genes (Minotto et al., 1999). The sequence TATAA, a putative TFIID/TBP binding element, was located $\approx$ 60 bp upstream from the start codon (Fig. 1). The sequence CAAAT box occurred two times within 90 bp upstream from the start codon (Fig. 1) that has been reported in the upstream sequences in other giardial genes (Minotto et al., 1999).

The FASTA homology comparison program of the BLAST search revealed a high degree of homology between *gicyp1* and cyclophilins of many other species. The identity with other Cyps was 58-71%. Gicyp1 shared 71% and 64% amino acid identity with *E. histolytica* cyclophilin (AF017993) and human cyclophilin (X52851), respectively. The predicted amino acid sequence has a calculated molecular mass of 19,005 Da. One motif YkgSxFHRXIpkFMiQGG (amino acid 59-76) corresponded to the Cyclophilin-type peptidyl-prolyl cis-trans isomerase signature in the Prosite library of sequences. The 13 residues that constitute the cyclosporin (CsA) binding site of human cyclophilin were also detected in the amino acid sequence of Gicyp1, including tryptophan residue essential for the drug binding (Liu et al., 1991) (Fig. 2).

Southern blot analysis

Genomic DNA of *G. intestinalis* WB strain was digested with three restriction enzymes, and the digests were separated on 1% agarose gel and transferred to nitrocellulose membrane. The filter was probed with the radioactively labeled partial *gicyp1* cDNA. As shown in Fig. 3A, the result of the

agaaagacgacatacngtttcttccatgttggngttgcaaatgacttgagtntgttggng -78

ttgcaaatgacttgag[tataa]cgagaacgttgatgcgaaccatgcctactggatctcttc -18

ccagcangccggcacgcgATGTGCGCTCAGCCTAGAATTACGGCCGCTGAATTCGTTTCT 42

M C A Q P R I T A A E F V S

*GACAA*GGTCTTCTTTGACATCACCATCGGCGGCAAGCTCTTCGGCAGGATTACCATGGGG 102

D K V F F D I T I G G K L F G R I T M G

CTCTTCGGCTCAATCGTCCCGAAGACCGCGGAGAACTTCAAGAAGCTCTGCACCGGCGAG 162

L F G S I V P K T A E N F K K L C T G E

ATGGGTTTCGGCTACAAGGGCTCTACTTTCCACCGCGTCATCCCCAAGTTCATGATCCAG 222

M G F G Y K G S T F H R V I P K F M I Q

GGTGGGGACTTCACGAACCATAACGGCACTGGCGGCAAGTCCATCTATGGCGCCAAGTTT 282

G G D F T N H N G T G G K S I Y G A K F

CCTGACGAGAACTTCGAGATCAAGCACTTCGTCGGGTCGCTCTCCATGGCGAATGCTGGG 342

P D E N F E I K H F V G S L S M A N A G

CCGAACACCAACGGCTCTCAGTTCTTCCTCACCGTGGCCGACACTGCTTGGCTCGACGGC 402

P N T N G S Q F F L T V A D T A W L D G

AAGCACGTTGTCTTCGGGCGTGTCCTTGACGGGATGGACGTCGTCAAGGCAATCGAGACT 462

K H V V F G R V L D G M D V V K A I E T

ACGAAGACCGGGGCCAACGACAAGCCGGTTGAGAAGGTTGTCATCGCCGACTGTGGCGTG 522

T K T G A N D K P V E K V V I A D C G V

CTCCAGTAAgccaa**agtaaa**tgcgctaaaaaaaaaaaaaaaaaaaaaaaaaaa 576

L Q -

Fig. 1. Nucleotide sequences and putative amino acid sequences of GicypI. Putative transcriptional signal sequence 'tataa' is shown as boxed sequence. The bolded and underline sequences 'agtaaa' indicate polyadenylation consensus sequence. The italic characters of nucleotide sequences were detected by 5′ RACE PCR analyses. The gray-boxed characters represent initiation signals in *Giardia*.

hybridization showed the presence of one large DNA band between 4 and 9 kb.

Northern blot analysis

Total RNA was isolated from *G. intestinalis* trophozoites and analyzed by Northern blotting with the radiolabeled 500-bp cDNA insert as a probe (Fig. 3B). The positive signal of approximately 700 bp consisted of a cDNA coding sequence of 576 bp, 5′ untranslated region, 3′ noncoding region, and a poly A tract.

Expression, purification, and enzyme activity of the recombinant protein

GicypI clone was digested with *Eco* RI/*Xho* I, and the insert was cloned into the prokaryotic expression vector pGEX4T-1 (pGX-

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G.int    ---------MCAQPRITAAEFVSDKVFFDITIGGKLFGRITMGLFGSIVPKTAENFKKLC
E.his    ----------MARP----------KVFFDITIGGEKAGRIVMELFNDIVPKTAENFRCLC
P.fal    ---------MSKRS----------KVFFDISIDNSNAGRIIFELFSDITPRTCENFRALC
B.mal    ----------MSRP----------KVFFDITIDGSNAGRIVMELFADIVPKTAENFRCLC
L.maj    MPYTPHYPVVESNP----------KVWMDIDIGGKPAGRVTMELFKDAVPQTAENFRALC
Yeast    ------------MS----------QVYFDVEADGQPIGRVVFKLYNDIVPKTAENFRALC
Mouse    ----------MVNP----------TVFFDITADDEPLGRVSFELFADKVPKTAENFRALS
Rat      ----------MVNP----------TVFFDITADGEPLGRVCFELFADKVPKTAENFRALS
Human    ----------MVNP----------TVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALS

G.int    TGEMGFG-------YKGSTFHRVIPKFMIQGGDFTNHNGTGGKSIYGAKFPDENFE----
E.his    TGEKGNGLT-----YKGCGFHRVIKDFMIQGGDFTRHNGTGGKSIYGTKFADEAFT----
P.fal    TGEK-IGSRGKNLHYKNSIFHRIIPQFMCQGGDITNGNGSGGESIYGRSFTDENFN---M
B.mal    TGERGMGRSGKKLHYKGSKFHRVIPNFMLQGGDFTRGNGTGGQSIYGEKFPDENFQE---
L.maj    TGEKGFG-------YANSPFHRVIPDFMCQGGDFTNGNGTGGKSIYGSKFADESFLGKAG
Yeast    TGEKGFG-------YAGSPFHRVIPDFMLQGGDFTAGNGTGGKSIYGGKFPDENFK----
Mouse    TGEKGFG-------YKGSSFHRIIPGFMCQGGDFTRHNGTGGRSIYGEKFEDENFIL---
Rat      TGEKGFG-------YKGSSFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFIL---
Human    TGEKGFG-------YKGSCFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFIL---

                                             *
G.int    IKHFV-GSLSMANAGPNTNGSQFFLTVADTAWLDGKHVVFGRVLDGMDVVKAIETTKTGA
E.his    VKHTKPGMLSMANAGPNTNGSQFFITTVPCPWLDGKHVVFGQVVEGYDVVKMIENNPTGA
P.fal    KHDQ-PGLLSMANAGPNTNSSQFFITLVPCPWLDGKHVVFGKVIEGMNVVREME--KEGA
B.mal    KHTG-LGVLSMANAGPNTNGSQFFICTAKTEWLDGKHVVFNRVVEGMNVVKAVE--SKGS
L.maj    KHFG-PGTLSMANAGPNTNGSQFFLCTAPTSWLDGKHVVFGQVLEGYEVVKAME--AVGS
Yeast    KHHDRPGLLSMANAGPNTNGSQFFITTVPCPWLDGKHVVFGEVVDGYDIVKKVE--SLGS
Mouse    KHTG-PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAME--RFGS
Rat      KHTG-PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMSIVEAME--RFGS
Human    KHTG-PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAME--RFGS
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Fig. 2. A comparison of the deduced amino acid sequences of *Giardia* cyclophilin with other cyclophilins. Bolded amino acid sequences indicate residues involved in the CsA binding. The tryptophan essential for CsA binding is indicated with an asterisk. G.int, *G. intestinalis*; E.his, *Entamoeba histolytica* (accession number AF017993); P.fal, *Plasmodium falciparum* (accession number U33869); B.mal, *Brugia malayi* (accession number U47811); L.maj, *Leshimania major* (accession number Y13576). Yeast, accession number X17505; mouse, accession number X52803; human, accession number X52851; rat, accession number M19533.

Gicyp1). As revealed by 12% SDS-PAGE (Fig. 4), the recombinant protein was expressed as a fusion protein of *G. intestinalis* Gicyp1 and glutathione-S-transferase with an expected size of 57 kDa. GST fusion recombinant protein was cleaved with thrombin, and the resulting protein was purified on a glutathione-sepharose column and found to be Mr = 20 kDa size (Fig. 4). Purified recombinant Gicyp1 was assayed for PPIase activity. The isomerization of the Ala-Pro bond coupled with chymotryptic cleavage of the trans peptide was measured by the increase of absorbance at 390 nm. Recombinant Gicyp1 protein clearly accelerated the rate of *cis* $\rightarrow$ *trans* isomerization of the peptide substrate relative to the control and the catalysis was completely inhibited by the addition of 0.5 μM CsA (Fig. 5).

DISCUSSION

Cyclophilin coding gene *gicyp1* was identified in *G. intestinalis* for the first time. The incomplete cDNA clone Gi054 lacked 5′ untranslated regions and start codon but had complete 3′ region including the stop codon and polyadenylation signal. Through 5′ RACE PCR, complete sequences of 5′ region were obtained. Yee et al. (2000) showed that the GDH promoter with AT-rich region at the initiation site as well as a CAAAT region 34 bp upstream was important. The results of 5′ RACE PCR revealed that the *gicyp1* gene also

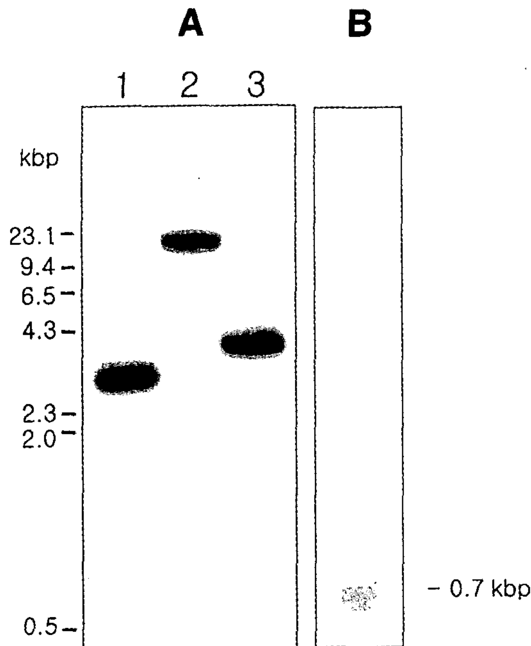

Fig. 3. Southern hybridization (A) and Northern hybridization (B) of *Giardia intestinalis* cyclophilin gene. (A) The genomic DNA of *G. intestinalis* were digested with *Pst* I (Lane 1), *Eco* RI (Lane 2), and *Hind* III (Lane 3).

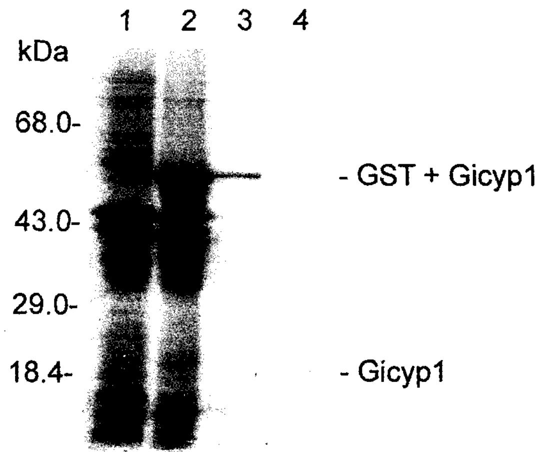

Fig. 4. SDS-PAGE gel electrophoresis patterns of recombinant GST fusion protein and purified Gicyp protein. Lane 1, crude extract of recipient strain *E. coli* BL21; 2, crude extract of transformed BL21 expressing Gicyp; 3, purified GST-Gicyp fusion protein; 4, purified Gicyp.

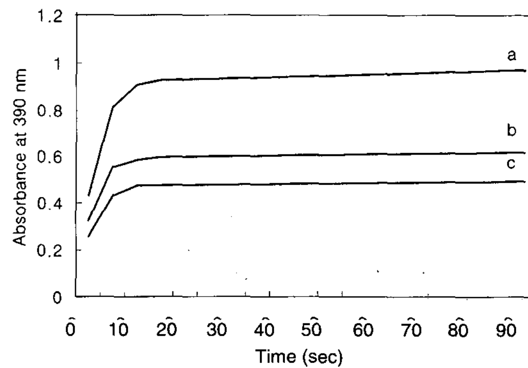

Fig. 5. *Giardia* Gicyp is an active and cyclosporin A - sensitive PPIase in protease-coupled peptide assay. The time course of *cis* $\rightarrow$ *trans* isomerase of *N*-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide coupled with chymotryptic cleavage was measured by the increase of absorbance at 390 nm using Shimadzu UV-1201 spectrophotometer. The data were collected every 5 s over 3-8 min interval. The isomerization of the peptide substrate (c; negative control) was accelerated in the presence of *Gicyp1* at 20 nM (a). This PPIase activity was blocked after incubation of 20 nM Gicyp with 0.5 μM CsA (b).

had this promoter region (Fig. 1). A motif resembling the putative AGTRAAY polyadenylation signal, often found in giardial genes (Minotto et al., 1999), was found 6 bp downstream from stop codon and 6 bp upstream from poly A sequences. These results are in agreement with the typically short 5′ and 3′ untranslated regions reported for other *Giardia* genes (Adam, 2001). The full-length coding sequence of Gicyp1 revealed 71% amino acid identity with previously reported *Entamoeba* cyclophilin EhCyp (Fig. 2). This extent of identity with EhCyp was higher than those of other parasites and some mammal Cyp sequences, the result being in agreement with the evolutionary lineage of *Giardia* and *Entamoeba*, which lack mitochondria. The Gicyp1 recombinant protein has deduced molecular weight of 19 kDa, which is within the range of other Cyps. The Cyp signature motif and residues involved in CsA binding were detected in Gicyp1 putative amino acid sequence. Also, the functional properties of the purified recombinant Gicyp1 demonstrated that it possessed PPIase activity and this activity was inhibited in vitro by CsA. The PPIase activity could be related with

FK506 binding protein and parvulins (Rudd et al., 1995); both did not bind to or were not inhibited by CsA, which is specific for cyclophilins (Trandinh et al., 1992).

The CsA has anti-parasitic effect that is well known however little has been known about its effect on *Giardia*. In this study, *G. intestinalis* was found to be susceptible to CsA in vitro, as most protozoan parasites were. The susceptibility of trophozoite PPIase to CsA correlated with the presence of essential tryptophan within the CsA binding site of Gicyp. A good correlation was observed between the concentration of CsA in the culture medium and the inhibition of trophozoite proliferation (data not shown). The 50% growth inhibits concentration (IC_{50}) of *E. histolytica*, *Plasmodium vivax* and *P. falciparum* were 1.0, 1.7, and 0.12-0.5 μg/ml, respectively (Reddy et al., 1995; Kocken et al., 1996; Ostoa-Saloma et al., 2000). Interestingly, some parasites were reported to be insensitive to CsA. IC_{50} values of *Brugia malayi* and *Toxoplasma gondii* were calculated to be 860 and 32.5 nM, respectively (Page et al., 1995). And, *Leishimania major* was not affected by concentrations less than 25 μg/ml. The differences among species in susceptibility to CsA may simply be due to differential drug uptake or different residue in place of the conserved tryptophan residue, which is essential to bind to CsA (Page et al., 1995).

The precise mechanism by which CsA exerts these anti-parasitic effects and the reason for the apparent species differences are yet to be elucidated. Nevertheless the mode of action may involve inhibition of the PPiase activity of endogenous cyclophilin or inhibition of an essential signal-transduction pathway, perhaps, via inhibition of a calcineurin homolog, which has been described for the immunosuppuression of T cells (Clipstone and Crabtree, 1992).

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