Anti-parasitic effect of cyclosporin A on *Echinococcus granulosus* and characterization of the associated cyclophilin protein

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SUMMARY

Cyclophilins are a family of proteins found ubiquitously in eukaryotes, many of which bind to the immunosuppressive drug cyclosporin A (CsA). CsA has been found to have anti-parasitic effects against a variety of helminth and protozoan parasites and this activity could be mediated via cyclophilin. In this study we characterize a full length cyclophilin gene from *Echinococcus granulosus*, the associated natural gene and expression pattern, and investigate the functional properties of the recombinant *E. granulosus* cyclophilin protein. In addition, the effects of CsA were investigated on *E. granulosus* protoscoleces in *in vitro* culture. The full length *E. granulosus* cyclophilin cDNA encodes a protein of 20 kDa and is encoded by a single gene (*EG*CyP-1) comprising 2 exons separated by a 31 bp intron. The gene is expressed constitutively in all *E. granulosus* life-cycle stages examined. Recombinant *E. granulosus* cyclophilin (*eg*CyP-1) exhibited functional enzyme activity as an isomerase. Treatment of *in vitro* cultures of *E. granulosus* protoscoleces with CsA was found to be lethal to the parasites. No protoscoleces survived treatment with 10 μg/ml of CsA over 7 culture days, as determined by observing motility and the uptake of toluidine blue dye. Untreated protoscoleces remained viable for the duration of experiments. The survival of protoscoleces was CsA dose dependent. A concentration of 10 μg/ml CsA was 100% lethal while doses of 8 μg/ml and 5 μg/ml resulted in 82% and 32% killing, respectively, after 7 days in culture. The anti-parasitic activity of CsA may have the potential to be developed as a new therapeutic agent for treatment of cystic hydatidosis in humans.

Key words: *Echinococcus granulosus*, hydatid, cyclophilin, cyclosporin A, isomerase.

INTRODUCTION

The tapeworm parasite *Echinococcus granulosus* is the causative agent of cystic hydatid disease in people and livestock animals. Humans infected with the larval stage of the parasite develop fluid-filled cysts, most often in the liver and lungs. While investigating the potential for recombinant antigens to be used for serological diagnosis of human hydatidosis, an antigen was identified which reacted prominently with sera from hydatid patients (Lightowlers, Haralambous & Rickard, 1989). The sequence of the associated cDNA revealed that the predicted protein had a high degree of homology with human cyclophilin.

Cyclophilins are a family of proteins that have been the subject of intensive investigation because they have been found to bind to the immunosuppressant drug cyclosporin A (CsA) (Marks, 1998; Hamilton & Steiner, 1998). CsA has been demonstrated to possess anti-parasitic effects on *Plasmodium* sp., *Trypanosoma* sp., *Eimeria* sp., *Toxoplasma* sp., *Schistosoma* sp. and some cestodes (Page, Kumar & Carlow, 1995*a*; Chappell & Wastling, 1992). The mechanism of action of CsA on these parasites is unclear and there is marked variability in the susceptibility to the anti-parasitic effects of CsA in different closely related species. *Hymenolepis microstoma* is killed both *in vitro* and *in vivo* by CsA but *H. diminuta* is unaffected (Chappell, Wastling & Hurd, 1989). Cyclophilin is present in *H. microstoma* and it binds CsA; however, attempts to date have failed to demonstrate CsA binding proteins or CsA sensitive isomerase activity in *H. diminuta* (Roberts, Sternberg & Chappell, 1995). *Echinococcus* species also vary in their susceptibility to CsA. *E. granulosus* parasites are adversely affected by the *in vivo* administration of CsA while *E. multilocularis* infection in mice has been reported to be relatively unaffected (Liance *et al.* 1992). When CsA was administered to mice that had been infected with *E. granulosus* protoscoleces, the establishment of secondary hydatid cysts was reduced both in terms of cyst number and weight (Hurd, Mackenzie & Chappell, 1993). However, other than these experi-

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ments, nothing is known about the susceptibility of *E. granulosus* protoscoleces or other life-cycle stages to CsA or the role that the cyclophilin protein may play in this drug susceptibility.

Cyclophilins have been isolated from a wide variety of eukaryotes and prokaryotes. They are a family of homologous proteins that bind to CsA and possess isomerase enzyme activity that catalyses the conversion of peptide substrates from *cis* to *trans* forms. Cyclophilins have been cloned from several parasites including *Schistosoma* sp. (Harding & Handschumacher, 1986; Argaet & Mitchell, 1992; Kiang *et al*. 1996; Bugli *et al*. 1998), *Plasmodium* sp. (Bell, Wernli & Franklin, 1994; Hirtzlin *et al*. 1995; Reddy, 1995; Berriman & Fairlamb, 1998), *Toxoplasma* sp. (High, Joiner & Handschumacher, 1994), *Leishmania* sp. (Rascher *et al*. 1998) and *Brugia* sp. (Page *et al*. 1995*a*; Page & Winter, 1998; Ellis *et al*. 2000). Different isoforms of parasite cyclophilins are present. *S. mansoni* has at least 3 genes encoding cyclophilins, SmCyPA (Argaet & Mitchell 1992), Sm17.7 (Kiang *et al*. 1996) with 62% homology to SmCyPA, and SmCyPB (Klinkert *et al*. 1995). Two isoforms have been described in *S. japonicum*, referred to as SjCyPA and SjCyPB (Klinkert *et al*. 1996). Both SjCyPB and SmCyPB possess a 23 amino acid hydrophobic leader sequence that is absent in SmCyPA and SjCyPA. Three cyclophilin genes have been cloned from *P. falciparum* (Hirtzlin *et al*. 1995; Reddy, 1995; Berriman & Fairlamb, 1998), *B. malayi* (Page *et al*. 1995*a*; Ma *et al*. 1996; Page & Winter, 1998), *Onchercerca volvulus* and *Dirofilaria immitis* (Ma *et al*. 1996; Hong *et al*. 1998; Hong, Ma & Carlow, 1998; Page & Winter, 1998), and 2 have been described from *T. gondii* (High *et al*. 1994). Each of these cloned parasite cyclophilins possesses the highly conserved CsA binding domain flanked by less conserved sequence and some isoforms have an amino-terminal signal sequence. Parasite cyclophilins do possess isomerase activity and bind CsA with varying affinities *in vitro* (Page *et al*. 1995*a*; Ma *et al*. 1996; Bugli *et al*. 1998; Rascher *et al*. 1998).

In this study, we characterize the full length *E. granulosus* cyclophilin cDNA, the associated gene and expression pattern and show that the expressed recombinant protein has functional PPIase activity. In addition, we demonstrate that cyclosporin A is lethal for *E. granulosus* protoscoleces in *in vitro* culture.

MATERIALS AND METHODS

Collection of E. granulosus *protoscoleces*

Hydatid cysts were collected from the liver and lungs of naturally infected sheep slaughtered at an abattoir in southern New South Wales. Protoscoleces were extracted aseptically from hydatid cysts sedimented and maintained for up to 5 days at 4 °C in cyst fluid. Viability of protoscoleces was assessed microscopically by observing morphology and flame cell activity.

The effect of CsA on E. granulosus *protoscoleces in* in vitro *culture*

Immediately prior to setting up *in vitro* cultures, protoscoleces were treated with 50 ml of artificial gastric fluid (1% pepsin plus 0·5 ml of 32% HCl made up to 100 ml in physiological saline) and gently mixed at 37 °C for 20 min. Protoscoleces were sedimented, the supernatant removed and exchanged with 50 ml of artificial intestinal fluid (Rickard & Bell, 1971) and gently mixed at 37 °C for 45 min. The percentage of evaginated protoscoleces was determined microscopically.

Activated protoscoleces were washed 3 times in 50× volume of sterile culture medium (Dulbecco's modification of Eagles medium, DMEM, Flow Labs, pH 7·4, filter-sterilized and supplemented with 100 I.U./ml mycostatin and 100 μg/ml gentamicin). DMEM plus antibiotics was added to each well of a 24-well sterile tissue culture plate (Nunc) under aseptic conditions. CsA (Sigma) (10 μg/μl in ethanol) was added to individual test wells to a final concentration of 10, 8, 5, 3, 1, 0·1, 0·01, 0·001 or 0·0001 μg/ml and the same volume of diluent alone was added to control wells. Approximately 500 evaginated protoscoleces were added to each of the culture wells, so that the total volume of media including CsA and protoscoleces was 2 ml in each well. Culture plates were incubated at 37 °C in 5% CO_2 humidified air. Culture media were changed every 48 h. The condition of the protoscoleces was assessed every 24 h. Motility and morphology was observed directly from culture dishes using an inverted microscope. Scores ranging between + (no movement) and +++ (rapid contracting movement), were assigned to quantitate relative activity. Parasite death was determined by collecting approximately 50 protoscoleces from each culture well per day and staining them with 0·1% toluidine blue in methanol (Vadas *et al*. 1979). The proportion of protoscoleces excluding the dye was recorded microscopically.

Cloning and expression of full length E. granulosus *cyclophilin*

In order to obtain full-length cDNAs for *E. granulosus* cyclophilin a lambda gt-11 cDNA library prepared from *E. granulosus* protoscolex mRNA (Lightowlers *et al*. 1989) was screened using EA21 cDNA that had been random primed and labelled with digoxigenine-11-dUTP (DIG-EA21) Boehringer, Mannheim). The insert size was determined for selected clones and those having the longest

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inserts were sequenced using the dideoxy termination method (Sanger, Nicklen & Coulson, 1977) with the ABI prism Big Dye termination cycle sequencing kit (PE Applied Biosystems). Sequencing reactions were electrophoresed and analysed on an ABI Prism 373XXL Sequencer. Both strands of double-stranded DNA were sequenced. DNA fragments were compiled and aligned using MacVector and AssemblyLIGN software (Oxford Molecular). The full cyclophilin coding sequence plus linkers 5′ *Bam*H1 to 3′ *Xho*1 were generated by PCR and the endonuclease-treated product was directionally ligated into the expression vector pGEX-2TEX. *Escherichia coli* BB4 cells were transformed and the transformants grown in Luria Bertani (LB) medium with 100 μg/ml ampicillin (AMP) overnight at 37 °C. Cells were diluted 1:10 in fresh LB/AMP and grown to log phase before being induced with 0·1 mM isopropyl thio-β-D-galactosidase. Following 4 h of growth at 37 °C, the cells were collected by centrifugation at 27000 ***g*** for 15 min, resuspended in phosphate-buffered saline (PBS) and sonicated 3 times for 30 sec. The glutathione-S-transferase (GST) fusion protein in the supernatant was bound to glutathione beads overnight at room temperature and washed 5 times with PBS. Bound fusion protein was eluted with glutathione, analysed by SDS–PAGE and stored at −20 °C. Enzymatic cleavage of cyclophilin from GST was performed while the protein was bound to glutathione beads by the addition of thrombin (1 unit/ml) (Amersham Pharmacia Biotech) to the washed beads. Following overnight incubation at 37 °C, the beads were sedimented and the supernatant was collected and analysed by SDS–PAGE.

Peptidyl prolyl cis–trans *isomerase assays*

Peptidyl prolyl *cis–trans* isomerase (PPIase) activity of *E. granulosus* cyclophilin was determined using the assay described by Fischer *et al*. (1989). The assay mixture contained 900 μl of buffer (50 mM Hepes, 86 mM NaCl, pH 8·0) plus 100 μl of 6 mg/ml chymotrypsin (Sigma). PPIase activity was assessed using 2 μg of thrombin-cleaved *E. granulosus* cyclophilin or using the same amount of human cyclophilin (Sigma) or GST as positive and negative controls, respectively. For each assay an additional control was performed in which only distilled water was added to the reaction in order to measure background colour produced in the absence of any added protein. The reaction was initiated by the addition of the peptide substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma), dissolved in 470 nM LiCl/trifluoroethanol (TFE) to a final concentration of 20 μM. The subsequent release of a yellow chromogenic dye was measured at 390 nm every 6 sec for 2 min using a Perkin Elmer MBA2000 spectrophotometer. All buffers, solutions and equipment were pre-equilibrated to 2 °C prior to commencement of the assay. The capacity of CsA to inhibit enzyme activity was determined using CsA (Sigma) dissolved in ethanol to 10 μg/μl. One μl of CsA stock was added to the assay mixture prior to the initiation of the reaction with substrate.

Southern blot analysis of E. granulosus *genomic DNA*

Ten μg of *E. granulosus* genomic DNA (gDNA) were incubated overnight with or without 10 units of *Eco*R1, *Bam*H1, *Hin*d111 or *Sac*1. Following the incubation, DNA was resolved in a 0·7% Tris-acetate EDTA (TAE) buffered agarose gel. Nucleic acids were transfered onto a positively charged nylon membrane and immobilized using UV light. The membrane was pre-hybridized for 5 h at 65 °C in 5 × SSC buffer, 0·1% *N*-lauroyl-sarcosine, 0·2% SDS, 1% blocking reagent (Boehringer Mannheim) then hybridized overnight at 65 °C using 5 ng/ml of DIG-EA21. Unbound probe was removed by washes at 2 × SSC and 0·5 × SSC. Positive hybridization was detected using anti-digoxigenin, Fab fragments conjugated to alkaline phosphatase (1:20000 dilution), a chemiluminescent substrate (Boehringer Mannheim) and exposure to X-ray film.

Cloning of the cyclophilin gene

DIG-EA21 was used to screen *E. granulosus* gDNA libraries in EMBL4 containing inserts in the size range 6–15 and 15–23 kilobases (Chow *et al*. 2001). Positive plaques were isolated and purified to a homogeneous stock. *Eco*R1 DNA inserts were restriction-digested from the vector, size separated on 1% TAE-buffered agarose gel and purified. The fragment was subcloned into PUC18, transformed into competent JM109 *E. coli* cells and grown in SOC medium for 1 h. Cells were plated out on LB agar plates impregnated with 100 μg/ml AMP, 0·5 mM IPTG and 80 μg/ml β-galactosidase. Recombinant colonies were picked and plasmid DNA prepared using standard alkaline lysis methods. Ligation of the insert into PUC18 was confirmed by *Eco*R1 restriction digestion and positive hybridization to DIG-EA21 in Southern blot. The approximate position of the cyclophilin gene on gDNA fragments was determined by restriction mapping of the gDNA fragment using a variety of 6-base restriction enzymes and hybridization to DIG-EA21 in Southern blot. DNA sequencing of the gDNA fragment was initiated using primers designed from the EA21 cDNA sequence.

Stage-specific expression of E. granulosus *cyclophilin*

RNA isolated from *E. granulosus* protoscoleces, oncospheres and adult worms (Puissant & Houde-

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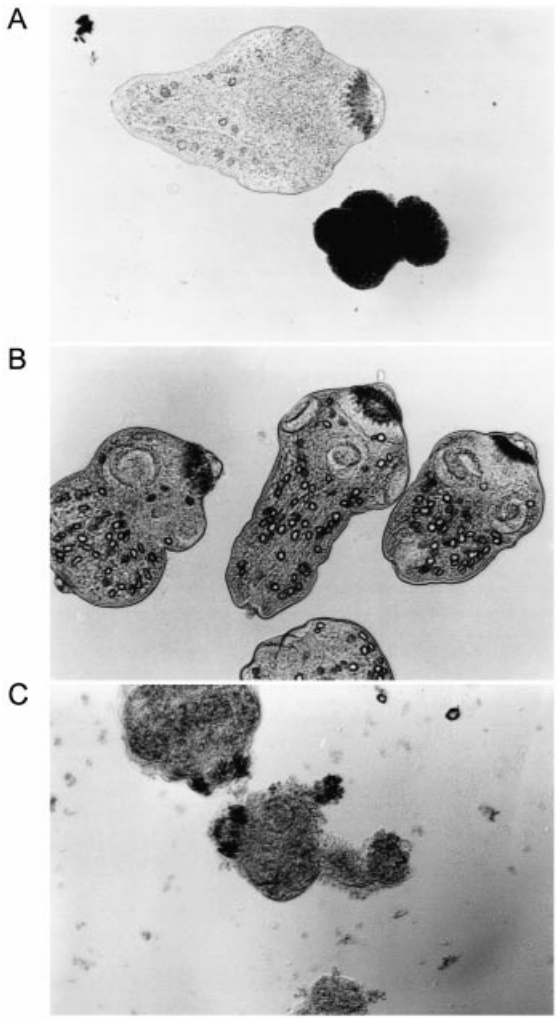

Fig. 1. The effect of exposure of *Echinococcus granulosus* protoscoleces to cyclosporin A *in vitro*. (A) Protoscoleces following 7 days culture and stained with toluidine blue. Non-viable protoscoleces were readily differentiated from unstained viable protoscoleces. (B) Viable protoscoleces following 7 days culture in the absence of cyclosporin A. (C) Non-viable protoscoleces following 7 days culture in the presence of 10 μg/ml of cyclosporin A.

bine, 1990) was reverse transcribed using oligo d(T) primers and superscript II reverse transcriptase (GIBCO BRL). Additional identical reactions were also included but without the inclusion of reverse transcriptase. Specific internal primers CYC1 (GC-GTGAAGTGCTTCTTCG) and CYC2 (GCAG-CCATCGTCATCACCGTTCTG) were designed to amplify a 420 base-pair product from *E. granulosus* cyclophilin. These primers were used in PCR on 1 ng of reverse transcribed RNA (or non-reverse transcribed RNA controls) to amplify cyclophilin genes in each life-cycle stage. PCR conditions were as described previously. PCR products were analysed on 1 % TAE-buffered agarose gel and nucleic acids were transfered onto nylon membrane for Southern hybridization to DIG-EA21. Southern blot conditions were as indicated above.

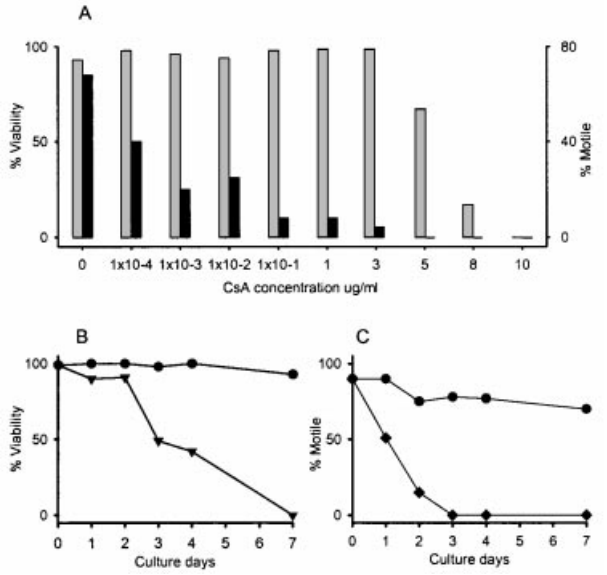

Fig. 2. Magnitude and time-course of the effects of cyclosporin A on *Echinococcus granulosus* protoscolex motility and viability. (A) Data recorded after 7 days *in vitro* culture in the presence of 0·0001–10 μg/ml of cyclosporin A. Effects on motility (score greater or equal to ++ motility – see Materials and Methods section) shown in black bars. Viability determined by exclusion of toluidine blue shown in grey bars. (B and C) Time-course of effects of culture with 10 μg/ml of cyclosporin A over 7 days culture period on protoscolex viability shown as (▼) and motility shown as (◆) respectively and untreated controls are represented by (●).

RESULTS

E. granulosus protoscoleces collected for *in vitro* culture were initially treated with pepsin, pH 2·0, followed by bile salts resulting in 95 % evagination. The protoscoleces appeared viable and highly motile. Non-viable protoscoleces could be identified by staining with toluidine blue whereas viable protoscoleces excluded the dye (Fig. 1).

CsA was toxic for *E. granulosus* protoscoleces in *in vitro* culture (Fig. 2). The toxic effect of 10 μg/ml of CsA on protoscoleces was observed from culture day 1 where 40 % of parasites showed no motility in comparison to > 95 % motility in control cultures. By culture day 7, treatment with 10 μg/ml of CsA killed all protoscoleces as determined by toluidine blue dye uptake. Susceptibility of protoscoleces to CsA was dose dependent. After 7 days in cultures containing 10, 8, 5 μg/ml of CsA the proportion of viable protoscoleces was 0 %, 18 % and 68 % respectively compared with control cultures in which 90 % of protoscoleces remained viable after 7 days. Lower concentrations of CsA impaired parasite motility (Fig. 2) but did not induce parasite killing over the 7 days in which the parasites were observed.

A full-length cDNA, corresponding to the EA21 clone, was isolated from the lambda gt-11 cDNA library (Genbank accession number AF430707

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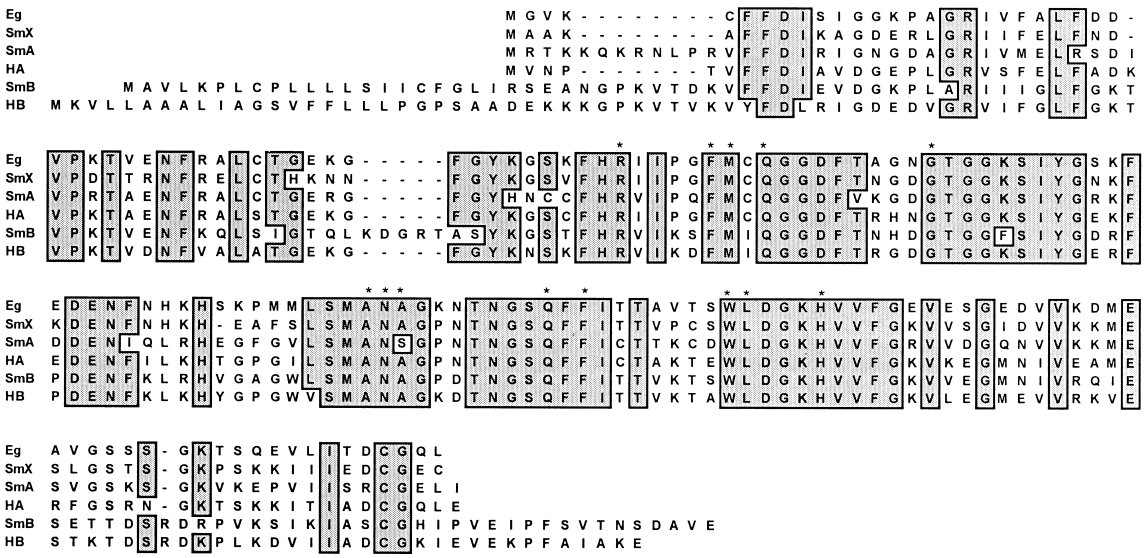

Fig. 3. Amino acid sequence alignment of *Echinococcus granulosus* cyclophilin (Eg) to other cyclophilins: SmX, *Schistosoma mansoni* Sm17.7 (Genbank accession number L46884); SmA, SmCyPA (U50388); HA, human CyPA (X52851); SmB, (U30874); HB, human CyPB M60857. The amino acid residues involved in CsA binding are marked (*).

modified from J04664) and designated *eg*Cyp-1. The EA21cDNA was found have been only a single nucleotide less than full length with respect to the coding sequence of the gene. A ribosomal binding site ATA was identified preceding the initiator methionine within a 49 bp 5′ untranslated region (UTR). The predicted initiation methionine aligned to the initiation codon of the cyclophilin A gene from *S. mansoni* (Klinkert *et al.* 1996) and humans (Haendler & Hofer, 1990). The 3′ UTR consisted of 127 nucleotides following the stop codon, including a polyadenalation signal sequence AATGAA at position 103–108. A poly $A_{(10)}$ tail and *Eco*R1 site were present at the 3′ end of the cDNA as would be predicted from the method by which the mRNA library was constructed.

Comparison of the predicted amino acid sequence of *eg*CyP-1 to the cyclophilin genes of other species (Fig. 3) indicated homology with other cyclophilins (71 % with *S. mansoni* Sm17.7 (Kiang *et al.* 1996), 63 % with *S. mansoni* SmCyPA (Klinkert *et al.* 1996), 65 % with *S. japonicum* SjCyPA (Argaet & Mitchell, 1992) and 71 % with human cyclophilin A (Haendler & Hofer, 1990)). Homology was lower with cyclophilins from the B subclass such as human cyclophilin B (55 % (Prince *et al.* 1991)) and *S. mansoni* SmCyPB (49 % (Klinkert *et al.* 1995)). The 13 amino acid residues of the highly conserved CsA binding domain (Hamilton & Steiner, 1998) were conserved in *eg*CyP-1 and the tryptophan residue described as critical for CsA binding to cyclophilins (Lui, Chen & Walsh, 1991) was present at position 119.

Affinity purification of *eg*CyP-1 GST fusion protein yielded a single band in 13 % SDS–PAGE at 43 kDa. Thrombin cleavage of the GST portion of the fusion protein immobilized on glutathione agarose released a protein of approximately 20 kDa into the supernatant, which corresponds to the predicted size of the parasite-encoded protein.

PPIase assays were performed using thrombin-cleaved *E. granulosus* cyclophilin *eg*CyP-1, human cyclophilin and GST. *eg*CyP-1 and the human positive control demonstrated PPIase activity (Fig. 4). In both cases the enzyme activity was inhibited by the addition of CsA. The assays shown in Fig. 4 utilized 2 μg of *eg*CyP-1. Assays undertaken using amounts of protein between 10 μg and 1 ng indicated that increasing amounts of *eg*CyP-1 corresponded with an increase in isomerase activity (data not shown). No PPIase activity was detected using recombinant GST as a negative control. In each experiment it was necessary to assess the level of autoisomerization that occurs due to a proportion of the substrate being in *trans* form at equilibrium. That portion is susceptible to cleavage by chymotrypsin forming a background colour unrelated to enzyme activity of added cyclophilin. Dissolving the substrate in 470 mM LiCl/TFE reportedly shifts the conformational equilibrium to the *cis* isomer by up to 70 % (Kofron *et al.* 1991).

Southern blot hybridization of restriction enzyme-digested *E. granulosus* gDNA with DIG-labelled EA21 cDNA revealed 1 band (Fig. 5). Screening of an EMBL4 *E. granulosus* gDNA library of the insert size range 15–23 kb with DIG-labelled EA21 cDNA isolated a clone containing a 20 kb *Eco*R1 insert showing specific hybridization to DIG-labelled EA21 cDNA. DNA sequence of that genomic clone, designated *EG*CyP-1 (Genbank accession number AF431734), revealed 100 % homology in the predicted exon sequence to the *eg*CyP-1 cDNA and a 31

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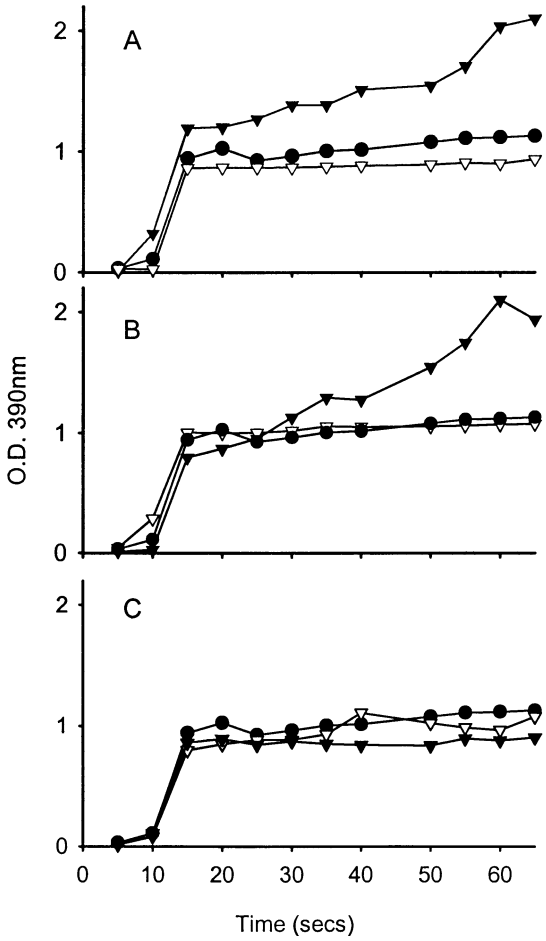

Fig. 4. Time-course of PPIase activity and inhibition of enzyme activity by CsA using the chymotrypsin-coupled chromogenic assay. (A–C) PPIase activity (▼) of *Echinococcus granulosus* cyclophilin, human cyclophilin and GST, respectively. (▽) Effect of CsA on the respective test protein; (●) negative control.

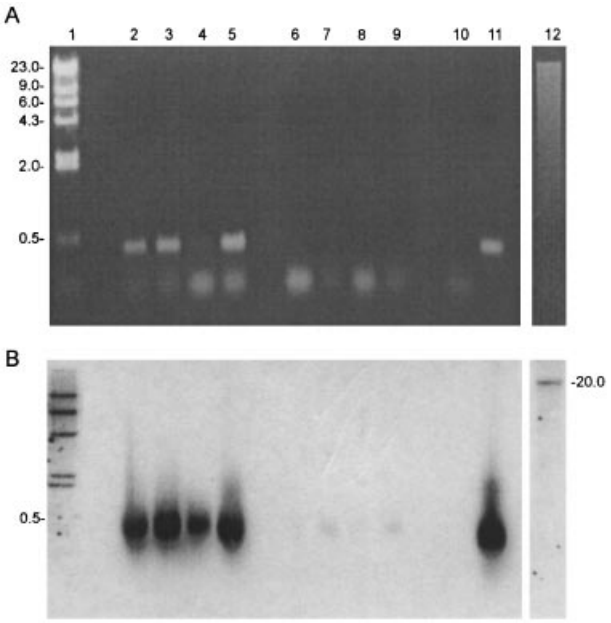

Fig. 5. RT-PCR and Southern blot analysis of the *Echinococcus granulosus* cyclophilin mRNA and associated gene. (A) PCR amplification of *E. granulosus* mRNA using cyclophilin specific primers CYC1 and CYC2 (see Materials and Methods section). Lane 1, DNA molecular weight markers; Lanes 2–5, mRNA template prepared by reverse transcription of RNA isolated from 4 life-cycle stages of *E. granulosus*; Lane 2, anterior segments (scolex, neck and first segment) of an adult worm; Lane 3, posterior (mature and gravid) segments of an adult worm; Lane 4, oncospheres; Lane 5, protoscoleces. Lanes 6–9 represent non-reverse transcribed PCR amplification controls; Lane 6, anterior segments adult worm; Lane 7, posterior segments adult worm; Lane 8, oncospheres; Lane 9, protoscoleces; Lane 10, PCR water control; Lane 11, EA21 cDNA positive control; Lane 12, *Eco*R1 digestion of *E. granulosus* genomic DNA. (B) Southern blot hybridization of samples in agarose gel (A) with DIG-labelled *E. granulosus* cyclophilin cDNA.

base pair intron at position 66 from the start codon. The intron was flanked by the nucleotides GT and AG similar to the dinucleotides which have been described as defining the boundaries of introns in other taeniid cestode genes (Waterkeyn *et al*. 1998). Additional sequence in the 5′UTR revealed a TAATAA box promoter element 56 base pairs upstream of the initiator methionine. Restriction mapping of *EG*CyP-1 indicated that the gene was positioned in the central portion on the 20 kb fragment. Hybridization of the DIG-labelled EA21 cDNA to *EG*CyP-1 restriction products indicate that the clone contains a single copy of the gene.

Following PCR amplification of reverse transcribed mRNA from *E. granulosus* protoscoleces, oncospheres and adult worms using *eg*CyP-1 cyclophilin specific primers, a single band was resolved by agarose gel electrophoresis in all stages (Fig. 5). No bands were present in the no-transcriptase controls. Southern hybridization of DIG-EA21 to the PCR products confirmed the specificity of the amplified product as a cyclophilin gene homologue. Some background was detected in the no transcriptase controls which may represent low levels of genomic DNA contamination in the mRNA sample.

DISCUSSION

CsA was found to be lethal for *E. granulosus* protoscoleces in *in vitro* culture. At concentrations of 10 μg/ml all protoscoleces were killed after 7 days exposure to the drug. Anti-parasitic effects were apparent within 24 h of exposure to CsA reflected in substantially reduced motility in the parasite compared with control cultures. The effect is substantially more pronounced than might have been suggested by the *in vivo* studies by Hurd *et al*. (1993) who found that while CsA did have an effect on the establishment of secondary hydatidosis in mice, the drug did not prevent the establishment of hydatid

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cysts. It is possible that the difference between the results obtained here in culture and those of Hurd *et al*. (1993) may reflect a quantitative difference in the concentrations of the drug to which the parasites were exposed and also the duration of exposure. In their experiments mice were inoculated subcutaneously with 50 mg/kg/day of CsA over 5 consecutive days. Variation in CsA dosage, the route and frequency of drug administration may result in greater parasite mortality *in vivo* than that observed by Hurd *et al*. (1993).

The anti-parasitic effect of CsA has been described in many parasites (Page *et al*. 1995*a*; Chappell & Wastling, 1992) and may be related to the inhibition of normal cyclophilin function following binding to CsA. Relatively little information is available about the biological role of parasite cyclophilins or their involvement in sensitivity of parasites to CsA. PPIase activity has been detected from all cloned parasite cyclophilins tested and from crude extracts of *H. microstoma* (Roberts *et al*. 1995). Other functions attributable to cyclophilins include the acceleration of protein folding and chaperone activity (Marks 1996; Gothel & Marahiel, 1999; Schiene-Fischer & Yu, 2001). Whether *E. granulosus* cyclophilin has an essential role in protein folding activity is yet to be established. However inhibition of normal protein folding by CsA may interfere with protein activity and affect parasite development as demonstrated here in *in vitro* culture.

More study of the biological activity of parasite cyclophilins and the anti-parasitic nature of CsA may potentially lead to the development of useful therapeutics. The finding that *E. granulosus* protoscoleces are killed by treatment with CsA may have implications for future treatment of human hydatid disease. Patients infected with hydatid cysts can potentially become reinfected by the escape of protoscoleces into the tissues of the body cavity during surgery. It may be possible that the introduction of CsA into a hydatid cyst during the PAIR surgical procedure (Filice *et al*. 1997) could be used to kill protoscoleces and potentially eliminate re-growth of hydatid cysts or the development of secondary cysts caused by the release of viable protoscoleces. Current scolicidal agents are not completely effective in killing protoscoleces (Filice *et al*. 1997) and development of a more potent scolicidal agent would be a valuable adjunct to the treatment of human hydatidosis.

The ability of cyclophilins to bind CsA can be predicted from the amino acid composition of the CsA binding domain. Cyclophilins generally possess a central, highly conserved, 13 amino acid domain originally identified through X-ray crystallography of human cyclophilin (Pflugl *et al*. 1993). The 13 amino acid CsA binding domain of *E. granulosus* cyclophilin is identical to that of the other parasite cyclophilins which have been shown to bind CsA. A tryptophan residue is present at position 119 corresponding to the tryptophan which has been shown to be critical for CsA binding by other cyclophilins (Lui *et al*. 1991). Most parasite cyclophilins cloned to date possess the tryptophan residue in the equivalent position. In the case of the *B. malayi* cyclophilins BmCyP-1 (Page *et al*. 1995*a*) and BmCyP-2 (Ma *et al*. 1996), isomerase activity of BmCyP-1 is relatively insensitive to inhibition by CsA (IC_{50} 860 nM) compared to the BmCyP-2 (IC_{50} 9·3 nM). The tryptophan residue of the CsA binding domain is conserved in BmCyP-2 but is replaced with a histidine in BmCyP-1. The crystal structure of BmCyP-1 revealed a conformational shift of the CsA binding site around the histidine residue in comparison with human cyclophilin A (Ellis *et al*. 2000). This structural change is suggested to reduce the CsA binding efficiency of BmCyP-1 (Ma *et al*. 1996). In the case of *E. granulosus*, no other isoforms of cyclophilin have been described to assess variations in CsA sensitivity resulting from sequence variation in the CsA binding domain. Further experiments to determine specific enzyme activity of *eg*CyP-1 and other *E. granulosus* cyclophilin isoforms and inhibition concentrations of CsA on enzyme activity may quantify the relative CsA sensitivity of different *E. granulosus* cyclophilins *in vitro*.

The results of cyclophilin-specific RT-PCR suggest that *E. granulosus* cyclophilin is constitutively expressed in all of the parasite's life-cycle stages. Southern blot studies indicate that the cyclophilin protein described here is encoded by a single gene. Research that has been undertaken on the cyclophilins of other organisms, including another platyhelminths, suggest *E. granulosus* would be likely to express other cyclophilins. More extensive investigations of the Southern blot conditions including hybridization undertaken at lower stringency may reveal other *E. granulosus* cyclophilin genes. Alternatively, use of degenerate primers designed from conserved regions of cyclophilin genes from other platyhelminth species may be an alternative approach to identification of other subclasses of *E. granulosus* cyclophilin genes.

The relationship between *E. granulosus* cyclophilin and the sensitivity of the parasite to the lethal effects of CsA is not clear. Certainly CsA has anti-parasitic effects in *in vitro* culture, but whether this is due directly or indirectly to binding with the cyclophilin characterized here is yet to be proven. Some studies have found no direct correlation between the anti-parasitic effects of CsA and parasite cyclophilin (Khattab *et al*. 1998; McLauchlan, Roberts & Chappell, 2000). For example, *H. microstoma* was exposed to CsA and 3 weakly or non-immunosuppressive CsA analogues. All induced parasite damage but only 2 bound to parasite cyclophilin (McLauchlan *et al*. 2000). In another study, a lack of correlation was found between CsA and CsA analogue-induced

parasite killing of *S. mansoni* and CsA inhibition of SmCyPB PPIase activity *in vitro* (Khattab *et al.* 1998). The association between the anti-parasitic effects of CsA and parasite cyclophilin is nevertheless worthy of further investigation for other parasites given that there is marked variability in the susceptibility to the anti-parasitic effects of CsA in different closely related species. A correlation may also be found between other cyclophilin isoforms and the effects of CsA on parasites. An association would also be consistent with the known mechanism of action of CsA in indicating the events leading to suppression of activity of T lymphocytes (Hamilton & Steiner, 1998). Whatever mechanisms of action of CsA on protoscoleces, the anti-parasitic effects may have practical application in the development of improved methods for therapy of human hydatidosis.

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