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A membrane-anchored *Theileria parva* cyclophilin with a non-cleaved amino-terminal signal peptide for entry into the endoplasmic reticulum

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Abstract

Recent studies suggest that peptidyl-prolyl isomerases of the cyclophilin family, that access the secretory pathway, can be involved in the interaction of parasitic protozoa with mammalian host cells. The amino acid sequence of a cDNA encoding a cyclophilin family member of the intracellular protozoan parasite of cattle *Theileria parva* contains a conserved C-terminal domain that exhibits 70% amino acid identity to cyclophilin proteins from other organisms, and a unique 60 amino acid novel N-terminal extension. Cell-free expression of the cDNA revealed a 26 kDa amino translation product, indicating expression of the N-terminal domain. The protein-coding region contains three short introns, less than 100 base pairs in length and Northern blot analysis demonstrates expression of a single 0.9 kb transcript in the piroplasm and schizont stages. The transcript is present in high abundance in the intra-lymphocytic schizont stage. The recombinant protein binds to immobilized cyclosporin A, a finding consistent with peptidyl-prolyl *cis-trans* isomerase function in vivo. A predicted N-terminal signal peptide was functional for entry into the eukaryotic secretory transport pathway in a cell-free in vitro transcription/translation system. The C-terminal cyclophilin domain was translocated across the membrane of the endoplasmic reticulum and the uncleaved signal peptide functioned as a membrane anchor.

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Keywords: *Theileria parva*; Cell-free expression; Signal-anchor sequence; Schizont

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1. Introduction

Theileria parva is an intracellular protozoan parasite (Class Sporozoa), which has a complex life cycle involving several intracellular stages within a tick vector, and subsequently in the lymphocytes and erythrocytes of a bovine host (Norval et al., 1992). The schizont stage, which resides in the cytoplasm of infected lymphocytes, causes East Coast Fever, a cattle disease characterized by induction of a leukemia-like syndrome in the infected lymphocytes resulting in massive proliferation of these cells (reviewed in Morrison et al., 1986; Dobbelaere and Heussler, 1999). Extensive phenotypic modification of the host lymphocytes as a result of parasite infection is presumed to be at least partially due to secretion of proteins by the schizont into the host cytoplasm (Nene et al., 2000). *T. parva* proteins containing predicted signal peptides for targeting to the secretory pathway are therefore of considerable interest in the context of parasite biology, and may also constitute candidates for development of recombinant vaccines that induce protective cellular immunity against the parasite due to their potential to access the host cytoplasm and hence the Class I MHC presentation pathway (Nene et al., 2000). *T. parva* has a relatively small and compact genome, and most introns so far characterized are relatively short (reviewed by Nene et al., 1998). East Coast fever is frequently lethal in exotic cattle and the disease is of considerable economic importance in endemic areas in Sub-Saharan Africa (reviewed in Norval et al., 1992).

Cyclophilins are a class of peptidyl-prolyl *cis*–*trans* isomerases, that are ubiquitous and highly conserved in eukaryotes (reviewed in Göthel and Marahiel, 1999). Cyclophilin proteins within the same cell can have different subcellular localizations and cyclophilin-like domains can be part of larger proteins, with distinct functions. For example, the yeast *Saccharomyces cerevisiae*, has seven different cyclophilin genes encoded within the genome. Recent studies suggest that host cyclophilins may contribute to disease progression in HIV infections (Sherry et al., 1998). There is also data indicating a role for cyclophilins secreted by protozoan parasites in host cell invasion (Moro et al., 1995) and immuno-suppression (Pelle et al., 2002). One location for the peptidyl-prolyl isomerase enzymes is the endoplasmic reticulum, an organelle that is a fundamental component of the eukaryotic secretory pathway. The secretory pathway is unusual in intracellular sporozoan protozoa in that, the golgi complex is not visible ultra structurally, and more than one mechanism may be involved, at least in the case of secretion by *Plasmodium* species located within erythrocytes (reviewed by Wiser et al., 1999; Mattei et al., 1999). We describe herein the cloning and functional characterization of the gene encoding a cyclophilin that is highly expressed in the lymphocyte-transforming schizont stage of *T. parva*, and present evidence suggesting that the protein is anchored in the membrane of the endoplasmic reticulum (ER).

2. Materials and methods

2.1. Isolation of cDNA and nucleotide sequencing

cDNA sequences of three trypanosomal cyclophilin A homologues (*Trypanosoma brucei* *brucei*, GenBank accession number AAB07896, *T. congolense*, no. AAB07894, and *T. vivax*,

no. AAB07895) were compared and forward primer ILO 2817 5'-TGCGTTATGCACTGG-TGAGAAG-3' and reverse primer ILO 2820 5'-CCGAAGACGACGTGTTTGCCGTC-3' were designed and used in a PCR reaction with total single-stranded cDNA from purified *T. parva* piroplasms as the template. A 0.4 kb PCR product generated from *T. parva* cDNA was cloned into vector pGEM-T (Promega) and sequenced using the fmol™ DNA sequencing system (Promega). Sequence analysis revealed high similarity between the 0.4 kb PCR fragment and cyclophilin A genes of other organisms. The PCR fragment was used to screen a *T. parva* piroplasm cDNA library in λ gt11 (described in Bishop et al., 1997) by hybridization in order to isolate a full-length *T. parva* cDNA clone. The sequencing strategy involved synthesis of oligonucleotides derived from acquired sequences as primers. Genomic and cDNA nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AF222790 and AF222791, respectively.

2.2. Southern analysis, RNA preparation and Northern blot analysis

Restriction enzyme digestions, agarose gel electrophoresis, blotting onto nylon membranes (Hybond N, Amersham), and hybridization of filters used routine methods (Sambrook et al., 1989). DNA probes were labeled using a Prime-it kit (Stratagene). Filters were sequentially washed in $2\times$ SSC/0.1% SDS, $0.1\times$ SSC/0.1% SDS at 65 °C. *T. parva* piroplasm, schizont-infected lymphocyte and sporozoite-infected tick salivary gland RNA was prepared according to Bishop et al. (1997). Northern blot analysis was performed as described (Pellé and Murphy, 1993).

2.3. Cell-free expression

The coding region including the stop codon was amplified by PCR from first strand piroplasm cDNA using primers ILO 5036 5'-CGC GGA TCC ATG CAT CTT AGA CAA TAC TC-3' and ILO 4197 5'-CGC GAA TTC TTA GAG TTG CCC ACA GTC-3', the product was digested with *Bam*HI/*Eco*RI and ligated into plasmid Bluescript II KS resulting in plasmid KS-CPH. The insert sequence was confirmed. KS-CPH was linearized with *Sal*I and in vitro transcribed with T7 RNA polymerase as described (Ebel et al., 1997). In vitro transcription mixture was in vitro translated in a wheat germ lysate system with or without dog pancreas rough microsomes (RM) as described (Ebel et al., 1997), except that the K⁺ concentration was reduced to 92 mM. To test for membrane translocation or insertion of protein products, accessibility to proteinase K (PK) was used as described (Ebel et al., 1997). Carbonate extraction analysis Fujiki et al. (1982) of RM after translation and inhibition of *N*-glycosylation by acceptor peptide benzoyl-Asn-Leu-Thr-*N*-maleimide (AP) for oligosaccharyl transferase was performed as described in Ebel et al. (1999).

2.4. Cyclosporin a binding assay

Affinity chromatography with D-Ser⁸-cyclosporin A covalently bound to a polyacrylamide matrix using Affigel 10 (BioRad) was modified from (Kieffer et al., 1992). After translation of cyclophilin with RM, RM were pelleted through a cushion containing 0.25 M

sucrose, 0.5 M KCl, 50 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethane sulfonic acid pH 7.5, 5 mM DTT, and 3 mM MgCl₂ (100,000 × *g*, 15 min, 4 °C). The RM pellet was lysed on ice for 30 min with buffer containing 0.3% sucrose monolaurate, 20 mM Tris–HCl pH 7.5, 120 mM NaCl, 1 mM EDTA, 20 mg/l phenyl methyl sulfonyl fluoride. The lysate was clarified by centrifugation and incubated with affinity gel equilibrated with lysis buffer for 17 h at 4 °C on a rocker. The resin was pelleted, the supernatant (non-bound fraction) was collected, the beads were washed with 14 volumes buffer containing 0.05% sucrose monolaurate, 20 mM Tris–HCl pH 7.5, 120 mM NaCl, 1 mM EDTA and eluted with 14 volumes wash buffer containing 20 μg/l cyclosporin A (CsA).

3. Results

3.1. Cloning and sequence analysis

A partial clone was obtained by PCR from piroplasm cDNA. The fragment was used to isolate a full-length clone from a piroplasm cDNA library (see Section 2.1). The 783 bp nucleotide sequence (GenBank accession AF222790) revealed an open reading frame potentially encoding a 227 aa protein, a 41 bp 3' untranslated region and a 12 bp poly A tail. Database searches showed that the C-terminal 165 aa were closely related to a wide variety of cyclophilins and constitute a complete cyclophilin domain. This region has 70% amino acid identity with a plant cyclophilin of *Arabidopsis thaliana* and 49% identity with a cyclophilin from *Theileria annulata* (Swan et al., 1996). Fig. 1 shows the complete sequence of the *T. parva* cyclophilin (on top) and its alignment to sequences or relevant sequence fragments (below in order of decreasing similarity) of other cyclophilins. For the *Plasmodium falciparum* cyclophilin 19, the 13 residues involved in binding of the ligand cyclosporin A have been determined (Peterson et al., 2000). All but the Met 68, which forms a van der Waals interaction, are identical in the *T. parva* sequence. The N-terminus of the putative translation product encoded by the *T. parva* cyclophilin open reading frame included a potential signal peptide for entry into the endoplasmic reticulum with a predicted cleavage after either aa 22 or 23 according to (Von Heijne, 1986; Nielsen et al., 1997).

A fragment of the *T. parva* cyclophilin gene (GenBank accession AF222791) was PCR-amplified from genomic DNA using primers ILO 3864 5'-CGC GGA TCC CAC AAT CAC CCC AAG CAT-3' and ILO 3866 5'-CGG GGT ACC TTA GAG TTG CCC ACA GTC-3' designed from the cDNA sequence. Comparison of the genomic sequence and the cDNA sequence demonstrated the presence of three short introns of 87, 64 and 92 bp, between bases 244 and 330, 423 and 486 and 734 and 825, within the *T. parva* cyclophilin coding sequence. All three introns contained the 'gt' and 'ag' 5' and 3' consensus splice sites, which are typical of eukaryotes.

3.2. Southern and Northern hybridization analysis

Southern blot analysis using a full-length *T. parva* cyclophilin gene probe and high stringency washing resulted in recognition of single restriction fragments in *T. parva* genomic DNA, digested with *Eco*RI (Fig. 2, panel A, lanes 1 and 2) and *Bam*HI (Fig. 2, panel B, lanes

```

1                                                                                               60
Tp  MHLRQYSWLLILFYQLLIFSSCLKFHNHPKHYSSFINNVLVLRHRKFFDLFSQTLPEMSK
At  *-----AS
Tg20 *-----P
Pf19 *-----**
Ta  *-----KFLVLK*F*LVSVALCRKPKPVE*SHKV

61                                                                                               118
Tp  RPRVYFDLTVGGAKAGRVPFELFSDVVPKTAENFRALCTGEKSTP--GNPLHYKGSTFHR
At  H*K*F**M*I**P**K*I*M**Y*T*K*T*****GVGRS*K**F***S***
Tg20 N**F**I*S*IDK*P**I*E****A*****G*GRS*K**Y***C*P***
Pf19 *SK*F**I*S*IDNS*N**I*I*****I*T*R*C*****IGSR-*KN*****N*I***
Ta  THH*HLEVQ*T*EKAT*E*TL*V*G*Y*L****V*N**V*S**E*T*IGD---KHYS*V*D*F***
#

119                                                                                              178
Tp  VIPHFTCQGGDFTNHNHTGGKSIYAKFEDENFTLKHDRPFLLSMANAGPNTNGSQFVFT
At  ***N*M*****K*G*****E*****ER**T*G*I*****A*****I*C
Tg20 I**Q*M*****R*M*****E**E**A**S**S**E*****I**
Pf19 I**Q*M*****I**G**S**E**R**S**T*****N*M**Q**G*****S**I**
Ta  ***N*M*V*A**I**V*G****S*I****D*M*****A**K*G-VIA**R*****Y*I*
    ## # #                                     ### # #

179                                                                                              227
Tp  TVVTQWLDGKHVVFGVEVVEGKDVVRAVEAVGTQSGKPTKPVVVEDCGQL..
At  **K*D*****Q****L**K*I*K*I*SS*****A***EISS
Tg20 **PCP*****K**A*Q*E**KMM**E*RSN*Q*KCA*EISS*****S.
Pf19 L*PCP*****K*I**M*N**E*M*KE*AK**YVKRS**I**T***E*..
Ta  **A*N****R*****L*L**E*Y*TL*Q*I**T**D****S*V*Q*T*I*K*S*KVKKL
    ## #

```

Fig. 1. *T. parva* cyclophilin sequence (Tp) and alignment to sequences of an *A. thaliana* cyclophilin (At; GenBank T50772), 20kDa *Toxoplasma gondii* cyclophilin (Tg20; B53522), *P. falciparum* cyclophilin 19 (Pf19; AAC41390.1), and *T. annulata* cyclophilin (Ta; AAC47321.1). The one letter amino acid code is used. Residue numbers for the *T. parva* cyclophilin are shown and gaps introduced to maximize alignment are indicated by dashes. Asterisks indicate residues that are identical. Residues demonstrated to be important for cyclosporin A binding of Pf19 (#) are indicated below the alignment.

1 and 2), suggesting that this cyclophilin gene was present as a single copy in the genome and not closely related to other *T. parva* cyclophilin genes. In addition four other restriction enzymes generated a similar result of recognition of a single fragment (data not shown). There was no hybridization to uninfected bovine cell DNA (Fig. 2, panels A and B, lane 3). A Northern blot of *T. parva* RNA derived from purified piroplasms, schizont-infected lymphocytes and sporozoite-infected tick salivary glands was hybridized with a full-length *T. parva* cyclophilin probe. This resulted in detection of a transcript of approximately 0.9 kbp in piroplasms (Fig. 3, panel B, lane 1) and schizont-infected lymphocytes (Fig. 3, panel B, lane 2). This indicated relatively high levels of transcription in the schizont stage. There was no detectable signal from 20 μ g of *T. parva* sporozoite-infected salivary gland RNA (Fig. 3, panel B, lane 3). However, reverse transcription PCR suggested that the *T. parva* cyclophilin is also transcribed in sporozoites, but apparently at low levels (data not shown).

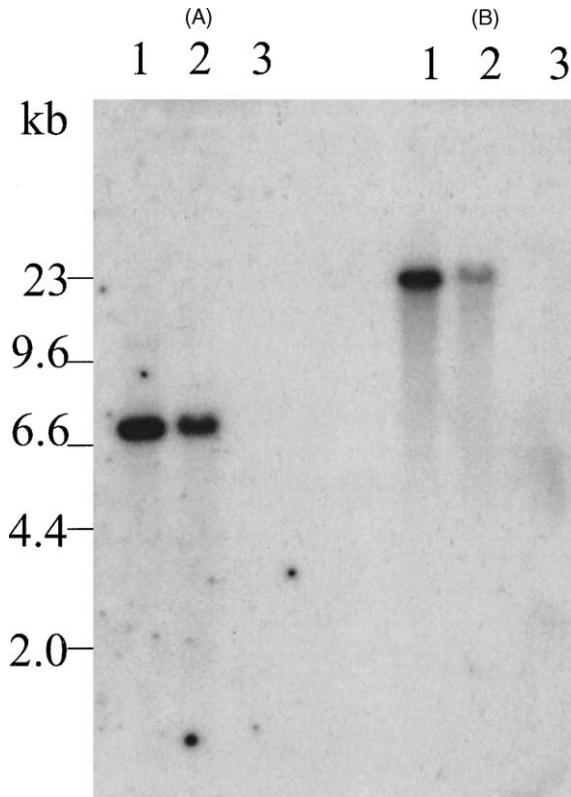


Fig. 2. Southern blot analysis of *T. parva* cyclophilin gene organization. Genomic DNA was digested with *Eco*RI (Panel A) and *Bam*HI (Panel B), size fractionated on an agarose gel and transferred onto a Nytran membrane. In each panel lane 1 contains purified piroplasm DNA (1 μ g), lane 2 contains *T. parva* schizont-infected lymphocyte DNA (20 μ g) and lane 3 contains uninfected bovine lymphocyte DNA (10 μ g). The filter was hybridized with a full-length radiolabeled *T. parva* cyclophilin cDNA probe and washed in 0.2 \times SSC/0.1% SDS at 65 $^{\circ}$ C. The mobility of the DNA size standards is indicated.

3.3. Analysis of endoplasmic reticulum membrane integration and processing

To analyze whether the putative signal sequence was functional, we expressed the cyclophilin cDNA in a coupled cell-free translation/translocation system and tested for processing and translocation of the protein across ER-derived membranes (Fig. 4). Translation in the absence of RM yielded a 26 kDa major product, in agreement with predicted product size (including the unique N-terminal extension) of 25,519 kDa. Smaller sized products are most likely due to premature termination. The 26 kDa product was completely sensitive to PK added after translation. When translation was performed in the presence of RM additional slower migrating products were observed. The processed forms were resistant to PK treatment unless detergent Nonidet-P40 (NP-40) was included to solubilize RM. A fraction of the 26 kDa product was digested by treatment with PK alone. This was not simply due to incomplete digestion of non-translocated protein, as carbonate extraction analysis

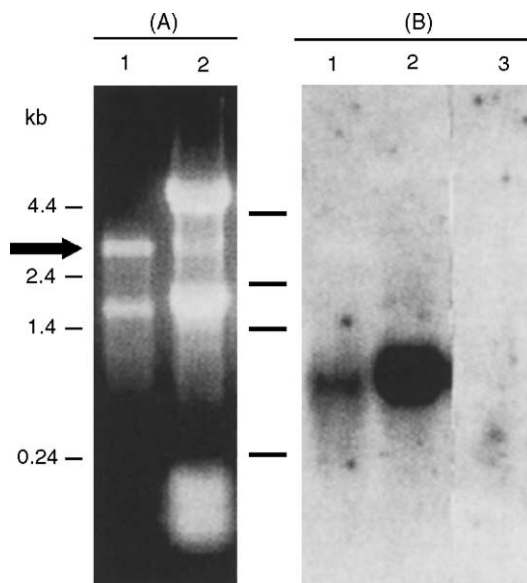


Fig. 3. Northern analysis of *T. parva* cyclophilin gene transcription. Panel A: total RNA from purified piroplasms (lane 1) and schizont-infected lymphocytes (lane 2) separated by agarose gel electrophoresis and stained with ethidium bromide. The ethidium bromide stained band corresponding to the parasite-specific large subunit rRNA is indicated by an arrow. Panel B: an autoradiograph of total RNA from purified piroplasms (lane 1), schizont-infected lymphocytes (lane 2) and sporozoite-infected tick salivary glands (lane 3), transferred to a nytran membrane (Amersham) after agarose gel size fractionation and hybridized with a radio-labeled full-length cyclophilin cDNA probe. The filter was washed in $0.2\times$ SSC/0.1% SDS at 65°C . The mobility of RNA size standards is marked.

revealed the occurrence of a membrane-integrated 26 kDa form (see below). In addition PK digestion was complete in control assays with a trans-membrane protein (not shown). The PK-protection analysis showed that RM-processing and translocation of the cyclophilin occurred and we conclude that the predicted signal peptide is functional.

We investigated whether the *in vitro* synthesized cyclophilin forms are integral membrane proteins using carbonate extraction of RM followed by centrifugation, where soluble and peripheral membrane proteins are found in the supernatant fraction and integral membrane proteins reside in the pellet fraction. When RM were pelleted first to remove the bulk of non-associated proteins and then fractionated, the processed forms and some of the 26 kDa form were found in the pellet (P), whereas the supernatant (S) contained only a trace amount of the 26 kDa form. When RM were digested with PK prior to carbonate extraction, the processed forms and some of the 26 kDa form were in the pellet fraction and the supernatant was devoid of undigested forms. Since carbonate extraction was controlled (not shown) we conclude that the RM-processed cyclophilin forms as well as a 26 kDa form are stably integrated into the ER membrane. Apart from the core of the signal peptide the sequence does not contain a hydrophobic stretch of sufficient length to anchor the protein in the membrane. Thus the protein sequence together with the carbonate extraction analysis suggested that the signal peptide was not cleaved off. The predicted proteolytic removal of

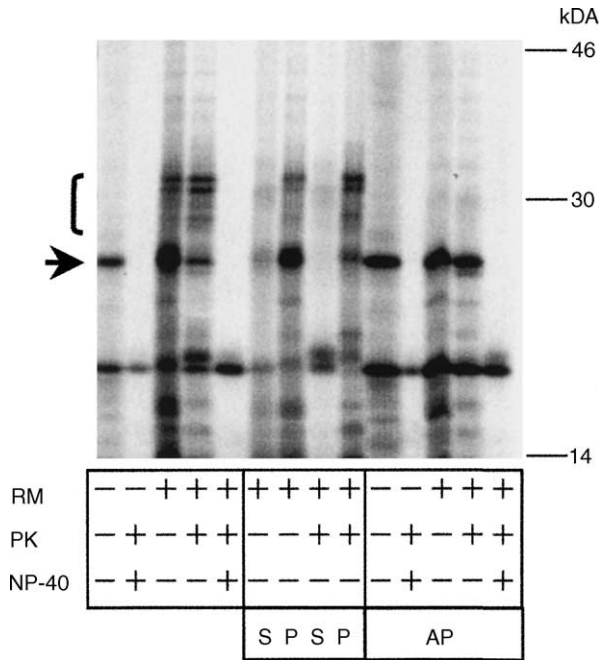


Fig. 4. In vitro expression analysis and processing of the *T. parva* cyclophilin. The cyclophilin was in vitro translated in a wheat germ lysate system either in the absence or presence of endoplasmic reticulum-derived membranes (RM) and acceptor peptide (AP) as indicated. For post-translational assays RM were pelleted from the reactions. Aliquots of the assays were post-translationally treated with proteinase K (PK) and detergent Nonidet P-40 (NP-40) as indicated. Aliquots of translation assays with RM were separated by carbonate extraction and centrifugation into supernatant (S) and pellet (P) fractions, either with or without prior PK treatment as indicated. Samples were analyzed by SDS-PAGE under reducing conditions and fluorography. The mobilities of the translation product in the absence of RM (arrowhead), of slower migrating RM-processed forms (bracket), and of size standards are marked.

the signal sequence would decrease the molecular weight by approximately 2.8–2.9 kDa. The cyclophilin has three potential *N*-glycosylation sites (residues 134, 150, 171) and addition of one *N*-glycan core in vitro results in an increase of apparent molecular mass of about 2.5–3 kDa. The pattern of RM-processed forms strongly suggested *N*-glycosylation of the protein, which would render detection of signal peptide cleavage in our gel analysis very difficult. Therefore, we tested for signal peptide cleavage by performing translation and RM translocation with inhibition of *N*-glycosylation (see Fig. 4). Under these conditions, the cleaved product should be well separated from the unprocessed 26 kDa form in our gel system. With AP and RM a large fraction of the 26 kDa form was resistant to PK digestion unless detergent was included, demonstrating that translocation occurred in presence of AP. Slower migrating RM-processed forms were not observed with AP, showing that they originated from heterogeneous *N*-glycosylation and glycan processing in the lumen of RM. A smaller sized form resulting from signal peptide cleavage was not detectable. However, the ability of this system to cleave *T. parva* signal peptide has been previously demonstrated (Ebel et al., 1997).

3.4. Binding to cyclosporin A

We asked whether the cyclophilin described here, including the novel N-terminal extension, can fold to allow specific binding to the undecapeptide cyclosporin A (CsA), a characteristic feature of most cyclophilins (Göthel and Marahiel, 1999). We expressed the cyclophilin *in vitro* and tested for specific binding to CsA covalently bound to a polyacrylamide matrix. In initial experiments with cyclophilin translated in the absence of RM, we could not detect binding (not shown). To separate membrane-translocated *T. parva* cyclophilin from untranslocated cyclophilin and the wheat germ lysate after translation, RM

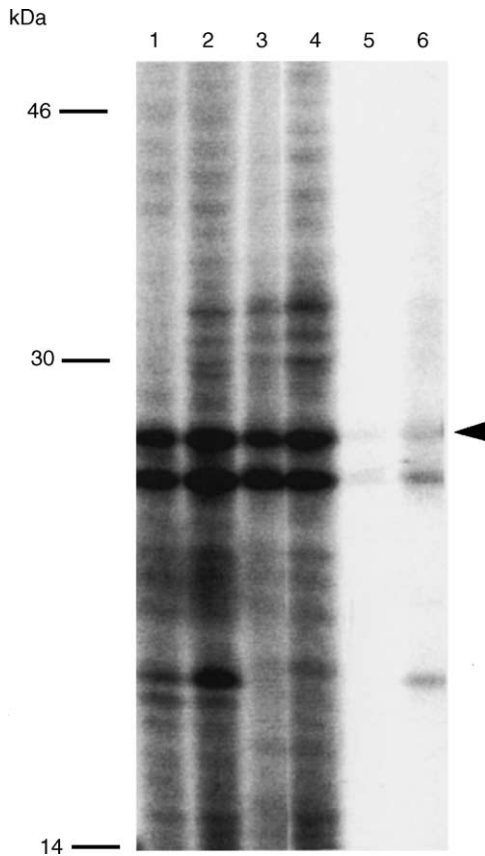


Fig. 5. Cyclosporin A (CsA)-binding to recombinant *T. parva* cyclophilin. The full-length cDNA was *in vitro* translated in a wheat germ lysate system either in the absence (lane 1) or presence (lane 2) of endoplasmic reticulum-derived membranes (RM). An assay with RM was split after translation, RM were pelleted (lane 3) or pelleted and solubilized with buffer containing 0.3% sucrose monolaurate. The lysate was incubated with immobilized CsA. The affinity resin was washed with buffer containing 0.05% sucrose monolaurate, and eluted with wash buffer containing CsA. The non-bound (lane 4), wash (lane 5), and eluted (lane 6) fractions were analyzed by SDS-PAGE under reducing conditions and fluorography. The mobilities of the 26 kDa-sized translation product (arrow) and the size standards are indicated.

were salt washed by centrifugation. The clarified detergent lysate of a RM pellet from a 250 μ l translation assay was incubated with 50 μ l affinity gel for binding. The resin was pelleted, the supernatant (non-bound fraction) was collected, the beads were washed and then eluted with wash buffer containing 20 μ g/l CsA. The fractions were concentrated by precipitation with trichloroacetic acid and analyzed by SDS-PAGE. As shown in Fig. 5, a fraction of the detergent-solubilized membrane-integrated 26 kDa form can be bound to immobilized CsA and specifically eluted with free CsA (lane 6). We could not detect specific binding of glycosylated cyclophilin forms, possibly due to too low amounts in the assay (see lane 3, representing the starting material). Two smaller sized proteins found in the eluate fraction are most likely either products of premature termination during in vitro translation or generated by proteolysis during the binding assay. The difference in the pattern of truncated translation products compared to Fig. 3 can be explained by use of a different preparation of wheat germ lysate. We could only bind a minor fraction of RM-integrated cyclophilin to the affinity resin. The detergent used for membrane solubilization seems to interfere with binding, since with higher concentrations the cyclophilin is almost exclusively found in the non-bound fraction (data not shown). Furthermore, the microsomes can be assumed to contain cyclophilins, which compete for CsA binding in the assay. We conclude that the *T. parva* cyclophilin binds specifically to CsA and hypothesize that it has peptidyl-prolyl isomerase function in vivo.

4. Discussion

A *T. annulata* gene encoding a cyclophilin that is transcriptionally up-regulated in the piroplasm stage has been described previously (Swan et al., 1996). Based on the low level of amino acid sequence identity, the N-terminal extension, and the relatively high levels of transcription in the schizont stage sufficient to allow detection by Northern blotting, it appears that the *T. parva* cyclophilin described here is not an orthologue of the *T. annulata* molecule, but a different cyclophilin family member. We were able to detect transcription by Northern blotting in both the schizont and piroplasm stages, however several examples of post-transcriptional regulation are known in *T. parva*. For example, both the PIM antigen and a 90 kDa heat shock protein are transcribed in the piroplasm stage, but their expression cannot be detected at the protein level by immunoblot analysis (reviewed by Nene et al., 2000). Given the high level of transcription of the *T. parva* cyclophilin observed in the schizont stage (Fig. 3) and the metabolic inactivity of *T. parva* piroplasms, which unlike those of *T. annulata* have not been shown to multiply in the erythrocyte, it seems more likely that the protein encoded by the gene we describe herein is functional in the schizont. The small size of the three introns we have identified in the cyclophilin gene, which are all <100 bp, provide further data indicating that certain *T. parva* introns are unusually short (Nene et al., 1998; Janoo et al., 1999). The *T. parva* cyclophilin contained an approximately 60 amino acid N-terminal extension that was not significantly similar to cyclophilin sequences in the public databases. Novel N-terminal extensions have been observed in other protozoan cyclophilin genes, including one recently described from *T. brucei* (Pelle et al., 2002). An N-terminal extension is also present in the predicted amino acid sequence of the gene encoding a *T. parva* casein kinase II subunit, which has a unique 99 amino acid

extension (Ole-MoiYoi et al., 1992). The function of such N-terminal extensions in otherwise evolutionarily conserved metabolic enzymes remains to be determined, however the *in vitro* translation and ER translocation data suggests that the novel domain in the *T. parva* cyclophilin is expressed *in vivo*.

Our analyses indicate that the *T. parva* cyclophilin is membrane-anchored via its non-cleaved signal sequence and that the C-terminal portion is translocated across the membrane as indicated by glycosylation in the cell-free system. This is the second *T. parva* protein for which *in vitro* functional analyses suggest the existence of an uncleaved signal peptide, the first being the p104 antigen (Ebel et al., 1999). The p104 protein is located in the membrane of the rhoptry, although initial post-translational processing presumably occurs in the ER, with subsequent translocation to the rhoptry. This type of N-terminal anchored membrane protein is believed to be relatively rare in nature, one well-characterized example being the influenza virus neuraminidase (Blok et al., 1982). However, similar *in vitro* transcription and ER translocation analyses demonstrated cleavage of the signal peptide in another *T. parva* protein, a secretory-type glutaredoxin (Ebel et al., 1997). In addition, whereas available algorithms allow relatively good prediction of N-terminal signal peptides for entry into the eukaryotic secretory pathway, the prediction of the cleavage is less reliable (Nielsen et al., 1997). Therefore, based on our data it is probable that this protein is targeted to the ER and becomes membrane anchored via an uncleaved N-terminal signal sequence, with the cyclophilin domain located in the organelle lumen. The question of glycosylation is not entirely certain, since *N*-glycosylation, although apparent in our *in vitro* assays has not yet been demonstrated for *T. parva* *in vivo* and *N*-glycosylation has been considered rare in sporozoan protozoa in correlation with apparent absence of morphological structures resembling a golgi apparatus (reviewed by Mattei et al., 1999). Whether the cyclophilin described here is located in the ER or transported to other cellular compartments is unknown. A number of cyclophilins entering the secretory pathway have been described, most of which are either soluble proteins located in organelles of the secretory pathway, or are secreted (Göthel and Marahiel, 1999). However, one well-characterized example of a membrane-anchored cyclophilin is the *Drosophila* NinaA protein. The post-translational processing of this protein involves translocation of the cyclophilin domain across the ER membrane, but in contrast to the *T. parva* cyclophilin, NinaA has a cleavable N-terminal signal peptide and a membrane-spanning segment close to the C-terminus (Stamnes et al., 1991). A likely function for a membrane-anchored *T. parva* cyclophilin would be chaperone activity within the secretory pathway as described for other family members. The high level of transcription in the intra-lymphocytic schizont stage of the parasite, which is actively involved in secretion of proteins into the host cell, is also consistent with such a role. However a definitive answer to the issue of specific function awaits the sub-cellular localization of the protein.

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