

Full Length Research Paper

Cloning and expression of *Theileria parva* T-complex 1 protein zeta subunit ortholog

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Accepted 2 September, 2009

East coast fever (ECF) is a severe lymphoproliferative disease of cattle caused by the intracellular protozoan *Theileria parva* from the family Apicomplexa. Gene homologs encoding antigens from other apicomplexan parasites constitute a source of vaccine candidate antigens. An ortholog of the zeta subunit of T-complex protein 1 (TCP-1) which plays a role in protein folding, assembly and transport was identified within the *Theileria parva* genome. The deduced amino acid sequence of the *T. parva* ortholog has a 55% identity and 77% similarity to the same protein in a number of eukaryotes such as *Danio rerio* (zebra fish) and man. The identity of this protein within its own family (*Apicomplexa*) is slightly higher. The *T. parva* TCP-1 zeta subunit gene was cloned and expressed in *Escherichia coli* and the recombinant protein was characterized.

Key words: T-complex protein, *Theileria parva*, apicomplexa, antigen.

INTRODUCTION

The chaperonins are a family of molecular chaperones involved in protein folding, assembly and transport. The chaperonin-containing T-complex protein 1 (TCP-1) with Cct subunits (Kubota et al., 1995) is abundant in the eukaryotic cytosol and is involved in the folding of actin and tubulin concomitant with ATP hydrolysis *in vitro* (Frydman et al., 1994). One of the characteristics that distinguishes TCP-1 from other chaperonins is its hetero-oligomeric nature, which in general comprises of eight different polypeptide species (Kubota et al., 1994).

Theileria parva is an apicomplexan that infects and transforms lymphocytes of cattle and African buffalo causing the disease called East Coast fever (ECF). Transmitted by *Rhipicephalus appendiculatus* ticks, the parasite causes a severe lymphoproliferative disease of cattle in eastern, central, and southern Africa (Katzer et al., 2006). It is an intracellular parasite that infects and transforms bovine lymphocytes. This has made it necessary for scientists to undertake research aimed at

developing a vaccine for ECF that is cheap, effective and easily available to the smallholder farmers who really depend on cattle as a means of survival.

Animals that recover from infection by *T. parva* have shown immunity to homologous challenge by the same by generation of parasite-specific cytotoxic T lymphocytes and also high titres of neutralizing antibodies after multiple exposures to the parasite. This has led to research that is aimed at developing a vaccine against the parasite. Initially, it was discovered that such immunity could be reproduced in cattle by infecting them with the parasite and treating them with tetracycline and theilericidal drugs (Radley et al., 1975). This method however is impractical as it requires the use of live parasites and it also does not guarantee cross-protection against different strains of the parasite. This has led to an approach that tries to determine the specific antigens that stimulate an immune response in infected animals and using them as targets in vaccines. These approaches include the action of neutralizing antibodies against p67 (the major sporozoite surface antigen) and the production of tumour necrosis factor alpha and interferon gamma by p67 specific CD4+ T-cells which

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inhibit the development of recently infected cells (Musoke et al., 1996).

The major mechanism responsible for the elimination of cells infected with the parasite is major histocompatibility complex (MHC)-class 1-restricted (parasite specific) CD8+ cytotoxic lymphocyte (CTL) activity. CTL activity though requires the input of specific CD4+ T-cell help for induction (McKeever et al., 1999). Katzer et al. (2007) observed that protective immunity against *T. parva* has considerable impact on the emergence of targeted parasites following challenge but fails to prevent their differentiation into transmissible forms. Furthermore, the genotypic compositions of the transmitted parasites arising from a given challenge vary between immune individuals with distinct MHC phenotypes.

It is therefore of great importance to focus on the identification of antigens of *T. parva* that are recognized by parasite specific CTLs. Six *T. parva* antigens, Tp1, Tp2, Tp4, Tp5, Tp7, and Tp8 recognized by CD8 T cells have been identified using CD8 T-cell lines from immune cattle to screen a parasite complementary DNA (cDNA) library and a set of cDNAs selected on the basis of bioinformatic analyses of the parasite genome (Graham et al., 2006, 2007). Homologs of apicomplexan antigens have been identified in the *T. parva* genome (TIGR) as possible antigen targets for a CTL response. Here we report an ortholog of one of these species, TCP-1 Cctz (zeta). This protein was also compared to the *Plasmodium falciparum* T-complex 1 protein zeta subunit.

MATERIALS AND METHODS

PCR and cloning

Polymerase chain reaction (PCR) was performed with a thermocycler (MJ Research, Watertown, MA) using *Taq* DNA polymerase (Promega) and two primers based on the sequences identified in *T. parva* using cDNA library as template (Graham et al., 2006). The PCR product generated above was cloned into pGEM T-easy vector (Promega, Madison, WI). Vector specific primers, both forward and reverse were synthesized as indicated; 5' GCCGCCACCATGGCAGTCAATATCTTAAATAGCAG 3' (forward primer) and 5'TTACGAAGGAGCGTTATGCATAGACCTT 3' (reverse primer). These primers were then used to amplify the TCP-1 ortholog gene from cDNA. All the PCRs were performed as described here in the following conditions: initial denaturation at 94°C for 3 min, 35 cycles of denaturation, 94°C for 1 min; annealing at 55°C for 1 min and polymerization at 72°C for 2 min. A final round of polymerization at 72°C for 10 min was performed at the end of the 35 cycles. Aliquots (10 µl) of PCR products with 5 µl loading buffer were loaded onto a 0.8% TAE agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

PCR products were extracted and purified using QIA quick Gel DNA Extraction Kit protocol (QIAGEN Co.) and the purified PCR products ligated into pGEM-T Vector (Promega Co., USA) according to the manufacturer's instructions. 1 µl of ligation reaction was transformed into *Escherichia coli* strain DH5-α competent cells. DNA nucleotide sequences were determined by gel based sequencing at the International Livestock Research Institute sequencing unit in Nairobi, Kenya. Sequences were analyzed using various basic alignment search tools (BLAST) served at the

National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Expression and purification of *T. parva* T-complex 1 protein zeta subunit

After sequence verification, part of the cloned fragment (722 bp) truncated portion was cloned into pET 28b, a bacterial based expression vector. The cloning sites used were generated by digesting with Hind III and XhoI. The construct of pET-TCP-1 was transformed into *E. coli* BL21 (DE3). For expression *E. coli* cells harbouring pET 28b with the cloned fragment, 500 ml of 2xYT bacterial broth was inoculated with 50 ml of overnight cultures of the respective *E. coli* transformed with homolog clones and grown in a 3 litre conical flask, in a 37°C rotatory incubator at 255 rpm (Becton Dickinson, Franklin Lakes, NJ, USA) to an optical density of 0.6 at 600 nm. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 2 mM and incubation for 24 h with time-lapse samples picked after 0, 3, 5 and 24 h. Bacterial cells were harvested, pelleted and weighed. Each batch of cells was then lysed with 5 ml of 8 M Urea, 0.1 M phosphate buffer pH 8.0 per 1 g of pellet, by gently stirring overnight at 4°C. This was then spun down at 10,000 g in a J100 Sorval rotor to get rid of cell debris. The supernatant was then mixed with 2 ml of 50% Ni-NTA agarose affinity matrix for 1 h, and the resin washed three times with 8 M urea-100 mM Na₂HPO₄-50 mM NaCl at pH 8.0 and once with the same solution at pH 7.5. Elution of bound recombinant proteins was performed by addition of 50 mM EDTA. Eluted proteins were analyzed for purity and molecular mass on a 12.5% SDS-PAGE gel, followed by Western blot analysis. The samples were then dialyzed against phosphate-buffered saline (PBS), passed through a 0.2 µm filter, and protein concentrations determined by a bicinchoninic acid protein assay (Pierce).

Western blot analysis

Expressed and purified proteins were confirmed using western blotting. 5 µg of each sample was run on a 12.5% SDS-PAGE gel at 35 mA in a 1 X running buffer (25 mM Tris, 250 mM glycine (electrophoresis grade) pH 8.3, 0.1% SDS prepared from a 10x running buffer stock solution). The transfer of proteins onto nitrocellulose sheets was performed as described by Towbin et al. (1979). After electrophoresis, the gel was equilibrated in transfer buffer (10% methanol, 24 mM Tris, 194 mM glycine) for 30 min to avoid any change in its size during transfer. The samples were electro-transferred onto nitrocellulose paper (0.45 µm protan nitrocellulose, Schelcher and Schuell, Dassel, Germany) at 70 V for 1 h at 4°C, or 15 V overnight, in transfer buffer. The blot was then stained with ponceau S (Sigma Aldrich, St. Louis MO, USA) to monitor the transfer, and then destained in transfer buffer and several rinses with water. The nitrocellulose filter was then blocked with blocking solution (TBS-Tween: 20 mM Tris-HCl, 200 mM NaCl, pH 7.4 containing 5% non-fat dry milk) for 1 h. The primary antibody, anti-His-tag protein (His-tag monoclonal antibody, Novagen, EMB Sciences, San Diego CA, USA), was added to the blocking solution at a dilution of 1:2000 with 0.02% NaN₃ and incubated overnight. The next morning the filter was washed 4 times with PBS/0.1% Tween 20, 10 min each. The second antibody, rabbit anti-mouse IgG HRP conjugate (Amersham International PLC, Aylesbury Buckinghamshire, England), was added at a dilution of 1:1500 in blocking solution and incubated for 3 h. The filter was then washed two times with PBS/0.1% Tween 20, then twice with PBS. The blot was developed by addition of the chromogenic substrate 3, 3'-diaminobenzidine (DAB; Sigma) in PBS at a concentration of 1 mg/ml in the presence of 0.1% (v/v)

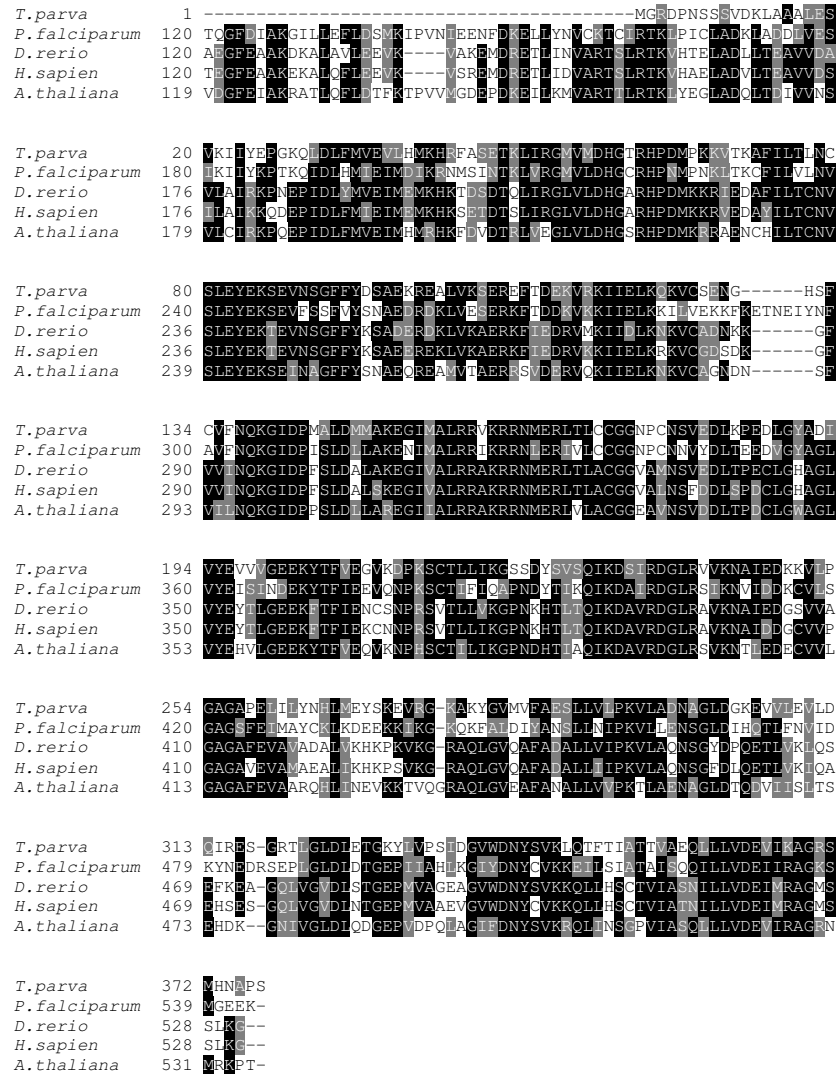


Figure 1. Amino acids alignment of TCP-1 with orthologs in other species. A comparison of deduced amino acid sequences of *T. parva* TCP-1 Cctz protein and its orthologs from *P. falciparum*, *D. rerio*, *A. thaliana* and *H. sapien*. The full length gene was truncated using Hind III and Xho I.

hydrogen peroxide. The filter was rinsed rapidly with water and dried sandwiched between two Whatman No. 3 mm filters.

SDS-PAGE

Total proteins as well as the purified *T. parva* T-complex 1 protein zeta subunit from the *E. coli* cells harbouring pET 28b with the cloned fragment were analysed by SDS-PAGE according to Laemmli (1970) using 12% polyacrylamide gels followed by staining with coomassie brilliant blue. Protein quantitative analysis was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Amplification by PCR, with a *T. parva* cDNA library as the

template, using the primers based on the signal peptide analysis described previously yielded a single 1,647 base DNA fragment. The full-length cDNA of *T. parva* TCP-1 Cctz contains an open reading frame encoding a predicted protein product of 548 amino acids with an estimated molecular mass of 60.71 kD and a pI of 6.07. The deduced amino acid sequence has a 55% identity with the TCP-1 Cctz protein of several organisms including *Plasmodium falciparum*, *Danio rerio* and man (Figure 1). The expression, purification and dialysis of the recombinant truncated protein from BL21 *E. coli* cells are shown in Figures 2 and 3.

The pathology associated with East Coast fever arises from invasion of lymphoid and non-lymphoid tissues by parasitized lymphoblasts. In a proportion of infected cells, the parasite undergoes further differentiation into

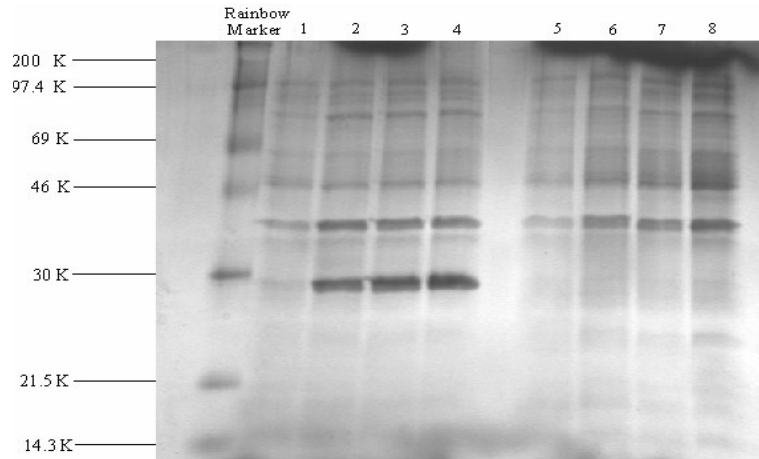


Figure 2. Protein expression from recombinant pET 28b clones. A 15% SDS-PAGE gel of the *T. parva* TCP-1 Cctz protein expressed in BL21 *E. coli* cells. The first four lanes were a time lapse induction of recombinant pET X9-3 cells: Lanes 1, 2, and 3 were protein extracts from the *E. coli* 0, 3, and 5 h post induction, respectively. Proteins in lane 4 were from an overnight induction. Lanes 5, 6, 7 and 8 were protein extracts from recombinant pET Y24b-1 cells and treated similarly as with recombinant pET X9-3 cells. The pET Y24b-1 cells showed no expression of recombinant proteins.

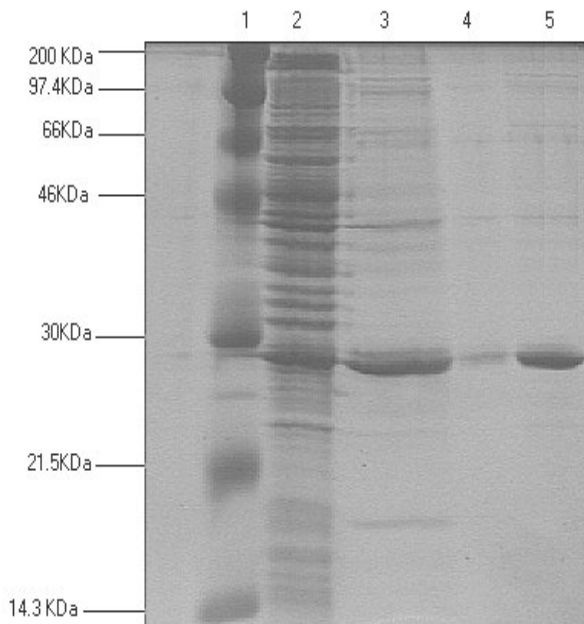


Figure 3. The purification and dialysis of the recombinant truncated *T. parva* TCP-1 Cctz protein from BL21 *E. coli* cells. A 15% SDS-PAGE gel of the purification and dialysis of recombinant pET X9-3. Lane 1 shows the rainbow marker. Lane 2 shows the lysate after denaturing *E. coli* cells in buffer containing 8 M urea. Lane 3 shows HIS-tagged protein after elution from the Ni-NTA agarose matrix. Lanes 4 and 5 are a result of 48 h of gradient dialysis in phosphate buffered saline. Lane 4 is an aliquot of precipitate in dialysed solution, whereas lane 5 is an aliquot of the dialysed protein solution.

uninucleate merozoite forms, which, upon release from the dying cell invade erythrocytes and develop into piroplasms, the infective stage for ticks. When ingested by a subsequently feeding tick, piroplasms give rise to gametes, which undergo syngamy in the gut lumen to form zygotes. These invade gut epithelial cells and differentiate into motile kinetes, which enter the hemocoel and migrate to the salivary gland. There, they invade specialized acinar cells and undergo further schizogonous division to form sporozoites (Katzner et al., 2007).

Graham et al. (2006) have observed that, with one exception, T cells from each animal identified only a single antigen, and T-cell lines from animals of different MHC genotypes tended to identify different antigens (Graham et al., 2006). The detection of responses to only one of the six antigens, in many cases focused on a single epitope, supports previous evidence that there is a dominance hierarchy in the antigens recognized in CD8 T-cell responses to *T. parva* (Graham et al., 2008).

The major challenge in the development of a vaccine against *T. parva* based on the induction of T-cell responses will be to generate responses that are effective against all parasite strains. This will require further screening for the identification of potential antigens to provide broad protection. The ultimate vaccine against *T. parva* will almost certainly need to incorporate multiple antigens and epitopes in order to confer protection in the genetically diverse outbred cattle population exposed to challenge by antigenically diverse parasite populations in the field (Graham et al., 2007).

ACKNOWLEDGEMENT

This work was done at the International Livestock Research Institute, Nairobi, Kenya.

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