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Short communication

The thrombospondin-related protein CpMIC1 (CpTSP8) belongs to the repertoire of micronemal proteins of *Cryptosporidium parvum*☆

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Abstract

Bioinformatic data show that, in addition to TRAP-C1, *Cryptosporidium parvum* encodes 11 thrombospondin-related proteins (CpTSP2 through CpTSP12), none of which has been characterized yet. We describe herein the cloning of a 2048 bp-long sporozoite cDNA encoding CpTSP8, a type I integral membrane protein of 614 amino acids, possessing three thrombospondin type I (TSP1) repeats and one epidermal growth factor (EGF)-like domain. Transcriptionally, *CpTSP8* is represented by a fully spliced and two immature mRNA forms, in which the intron is either totally or partially retained. Immunofluorescence analysis detected CpTSP8 in the apical complex of both sporozoites and type I merozoites, and showed that, upon sporozoite exposure to host cells *in vitro*, the protein is translocated onto the parasite surface as typical of micronemal proteins (MICs). Accordingly, double immunofluorescence localized CpTSP8 to *C. parvum* micronemes, prompting us to rename it CpMIC1 in agreement with the current MICs nomenclature.

Keywords: *Cryptosporidium parvum*; Invasion; Micronemes; MIC proteins; Sporozoite; Thrombospondin type I repeat

Among protozoan parasites, apicomplexans are unique for possessing a repertoire of extracellular proteins [1] containing adhesive amino acid domains typical of metazoans, e.g., the thrombospondin type I (TSP1) repeat, the epidermal growth factor (EGF)-like, the Apple and the von Willebrand Factor domains. Notably, the vast majority of parasite proteins belonging to this class of molecules have been localized to the micronemes [2], a class of apical secretory organelles characteristic of apicomplexan zoites. Furthermore, molecular and biochemical studies have shown that micronemal adhesins (MICs) are essential in host cell attachment/invasion and gliding motility [3,4]. A prominent role in these processes is played by proteins of the TRAP family [5], a group of transmembrane (TM) MICs identified in different genera of apicomplexans, containing at least one copy of the TSP1 repeat and sharing structural similarity with the thrombospondin-related anonymous protein (TRAP) of *Plasmodium* spp. [6]. Several lines of evidence support a direct role of the TSP1 repeats of TRAPs in the binding to sulphated glycosaminoglycans (GAGs) on the host cell surface [5].

We have previously characterized the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) [7], a TM micronemal adhesin of *C. parvum* sporozoites containing 6 TSP1 repeats. More recently, a bioinformatic analysis of the *C. parvum* genome predicted 11 additional proteins [8], named CpTSP2 through CpTSP12, possessing one to multiple copies of the TSP1 domain. Expression analysis of *C. parvum*-infected HCT-8 cells showed that the levels of individual *CpTSP* mRNAs

Abbreviations: EGF, epidermal growth factor; GAG, glycosaminoglycan; gDNA, genomic DNA; mAb, monoclonal antibody; MIC, micronemal protein; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; 5′-RACE, 5′-rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; TM, transmembrane; TRAP, thrombospondin-related anonymous protein; TRAP-C1, thrombospondin-related adhesive protein of *Cryptosporidium*-1; TSP, thrombospondin; TSP1, thrombospondin type I.

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AF061328.

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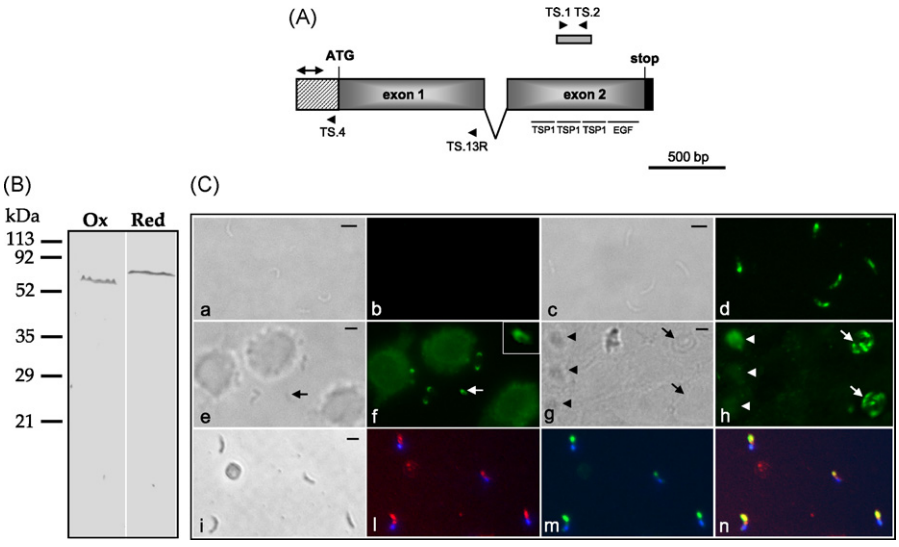

Fig. 1. (A) Structural organization of the *CpTSP8* gene. The coding sequence, 5′ UTR and 3′ UTR are represented as grey, hatched and black boxes, respectively. The arrowheads represent the primers used throughout the work, the double headed arrow indicates the 5′-RACE extension product. The grey horizontal bar above exon 2 corresponds to the genomic amplicon *Cp.amp3*, which was PCR amplified using the degenerate primers TRM.1–7 and TRM.1–7rev [7]. The position of the TSP1 and EGF-like domains encoded by exon 2 is indicated. The 5′ end of the *CpTSP8* mRNA was characterized using the SMART RACE kit (Clontech BD). Briefly, 1 μg of total sporozoite RNA was reverse transcribed with the oligonucleotide TS.13R (5′-TGAAAGGTTGACCTGGATTATC-3′), followed by first strand cDNA amplification with the universal anchor primer UPM and the nested gene-specific primer TS.4 (5′-CCCTTCACTGGTAATATCTTTCCATGC-3′). The amplicon was cloned into pGEM T-vector (Promega) and sequenced. (B) Immunoblot analysis. *Cryptosporidium parvum* proteins were extracted from excysted oocysts (Moredun) by four freeze–thaw cycles, followed by 10 min boiling in SDS-PAGE sample buffer. Oxidized (Ox) or β-mercaptoethanol-reduced (Red) proteins (5×10^6 oocysts/0.5 cm-wide slot) were separated on a 12% SDS-PAGE gel, blotted onto nitrocellulose and probed with the mouse antiserum SP1 (1:1000), raised against an histidine-tagged fragment of CpTSP8 (positions 181–586) produced in *E. coli*. Protein bands were visualised colorimetrically using an alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma). (C) Immunolocalization of CpTSP8. Following incubation of *C. parvum* oocysts with 1.5% sodium taurocholate, freshly excysted sporozoites were air-dried onto glass slides, fixed with 1% formaldehyde and permeabilized, if required, with 0.5% Triton X-100. Non-permeabilized (a, b) or permeabilized (c, d) sporozoites were incubated with serum SP1 (1:400) followed by a FITC-conjugated anti-mouse IgG secondary antibody (Sigma). Permeabilized parasites (c, d) show staining of the apical region and of an area anterior to the nucleus; (e–h): human HCT-8 cells infected with one freshly excysted oocyst per cell were fixed and permeabilized, if required, at 2 and 72 h p.i. and treated with serum SP1; (e, f): in non-permeabilized, extracellular sporozoites incubated for 2 h with HCT-8 cells, CpTSP8 appears to be apically located on the zoite surface. The arrow indicates the sporozoite shown in the inset at a higher magnification; (g, h): in HCT-8 cells 72 h p.i., type I merozoites (arrows) are apically labeled, whereas other parasite stages (arrowheads) show a diffuse staining or are negative. Extracellular and intracellular parasites treated with preimmune serum showed no fluorescent labeling (not shown); (i–n): fixed and permeabilized sporozoites were incubated with serum SP1 (l) and with the biotin-labeled mAb TOU (m) [12]. The merged image (n) shows co-localization of CpTSP8 and the micronemal antigen recognized by TOU. Briefly, the sporozoites were incubated with serum SP1 followed by a TRITC-conjugated anti-mouse IgG secondary antibody (Sigma). After extensive washing, the parasites were treated with the mAb TOU, labeled using the biotin hydrazide method (Pierce), followed by FITC-conjugated streptavidin (Sigma). Parasite nuclei were stained with 4′-6-diamidino-2-phenylindole. Samples were observed under a Zeiss Axioplan 2 epifluorescence microscope and images collected with an Axiocam digital camera (Zeiss). Scale bars = 2 μm.

are developmentally regulated [8], however, due to the non-synchronous growth of *C.* parvum *in vitro*, the study could not address the stage-specificity of individual transcripts, and also the localization of the corresponding proteins remains unknown. We describe herein the cloning of the cDNA encoding CpTSP8 and the localization of this protein in *C. parvum* zoites.

Using degenerate primers based upon the consensus sequence of the TSP1 repeat, we cloned the *TRAP-C1* gene [7] and a partial gDNA sequence named *Cp.amp3* (Fig. 1A), derived from the *CpTSP8* gene [8]. The *Cp.amp3*-specific primers TS.1 (5′-ACGGAGGGCTTACAATCAGGAC-3′) and TS.2 (5′-GAGAGAACACAATCGACTGGGCT3-′) were next employed to demonstrate by RT-PCR (not shown) that *CpTSP8* was expressed in *C. parvum* sporozoites and to screen a cDNA library from this stage by a PCR-based method, which yielded a poly-adenylated cDNA encompassing a 1842 bp-long open reading frame. Using the 5′-RACE technique, the *CpTSP8* mRNA was further extended by 119 bp, yielding a combined sequence of 2048 bp (GenBank accession no. AF061328) which included a 175 bp-long 5′ UTR and a 3′ UTR of 28 bp lacking a typical poly-adenylation signal (Fig. 1A). The second in-frame ATG (positions 176–178), lying 69 bp downstream of an in-frame stop codon, is most likely the translation initiation site, as predicted by the Sigcleave program (http://smart.embl-heidelberg.de). Consistent with previous RT-PCR data [8], alignment of the cDNA and genomic sequences confirmed that *CpTSP8* contains an intron, which in the Moredun strain consists of 222 bp. We have previously reported [9] that the *CpTSP8* intron harbours a highly polymorphic microsatellite, on the contrary, sequence analysis of 8 *C. hominis* isolates derived from England and Australia (not shown), showed that in this species the *CpTSP8* microsatellite is absent.

The screening of the cDNA library and RT-PCR analysis (not shown) revealed also the existence in *C. parvum* sporozoites of two immature *CpTSP8* mRNAs. In one of the two transcript variants, only the first 45 nucleotides of the intron were spliced out, whereas in the second the intervening sequence was totally retained. Alternative splicing based on intron retention