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Cryptosporidium parvum Genes Containing Thrombospondin Type 1 Domains

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Cryptosporidium parvum **is recognized as an enteropathogen of great worldwide medical and veterinary importance, yet understanding of its pathogenesis has been hampered in part by limited knowledge of the invasion machinery of this parasite. Recently, genes containing thrombospondin type 1 (TSP1) domains have been identified in several genera of apicomplexans, including thrombospondin-related adhesive proteins (TRAPs) that have been implicated as key molecules for parasite motility and adhesion onto host cell surfaces. Previously, a large-scale random survey of the *C. parvum* genome conducted in our laboratory revealed the presence of multiple genomic DNA sequences with a high degree of similarity to known apicomplexan TRAP genes. In the present study, TBLASTN screening of available *C. parvum* genomic sequences by using TSP1 domains as queries identified a total of 12 genes possessing TSP1-like domains. All genes have putative signal peptide sequences, one or more TSP1-like domains, plus additional extracellular protein modules such as Kringle, epidermal growth factor, and Apple domains. Two genes, putative paralogs *Cp*TSP8 and *Cp*TSP9, contain predicted introns near their amino termini, which were verified by comparing PCR products from cDNA versus genomic DNA templates. Reverse transcription-PCR analysis of transcript levels reveals that *C. parvum* TSP genes were developmentally regulated with distinct patterns of expression during in vitro infection. TRAPC1, *Cp*TSP3, and *Cp*TSP11 were expressed at high levels during both early and late stages of infection, whereas *Cp*TSP2, *Cp*TSP5, *Cp*TSP6, *Cp*TSP8, and *Cp*TSP9 were maximally expressed during the late stages of infection. Only *Cp*TSP4 was highly expressed solely at an early stage of infection.**

Although its association with human diseases was not perceived until 1976 (18, 27, 31), the apicomplexan parasite *Cryptosporidium parvum* has emerged as a significant pathogen both of humans and animals of veterinary importance. The parasite primarily infects the epithelial cells of the small intestine and causes gastrointestinal diseases in both immunocompetent and immunocompromised humans and animals. Typically self-limiting in immunologically healthy individuals, cryptosporidial infection can be persistent and life-threatening in hosts with impaired immune systems. Prolonged infection is compounded by the fact that there is currently no effective anticryptosporidial drug (19). Efforts to develop novel therapeutic strategies have been hampered by a lack of understanding of *C. parvum* pathogenesis and a paucity of stage- and organelle-specific markers with which to dissect the parasite's life cycle.

Thrombospondin-related adhesive proteins (TRAPs) have been identified in several genera of apicomplexans, including TRAP (38, 39, 45, 53) and CTRP (13, 54, 57, 60, 61) of *Plasmodium*, Etp100 of *Eimeria* (10, 32, 56), MIC-2 of *Toxoplasma* (2, 20), and NcMIC2 of *Neospora* (24, 46) spp. TRAPs are characterized by the presence of two adhesive modules: one or more von Willebrand factor A (vWA)-like domains, and one or more thrombospondin type 1 (TSP1)-like modules. TSP1-like domains were first identified as "region II" within the *Plasmodium* circumsporozoite protein (12) which, after the isolation of *Plasmodium* TRAP (36), was identified as a domain also present in thrombospondin and properdin. A series of studies describe a critical role for TSP1-containing proteins as mediators of host-parasite interactions and in the gliding motility of the parasite (28, 29, 47, 51). Secretion of *Toxoplasma* MIC-2 is associated with *Toxoplasma* invasion of host cells (7, 8), and disruption of the *Plasmodium berghei* TRAP gene resulted in sporozoites that displayed an impaired ability to invade mosquito salivary gland tissue and rat liver cells (52). TRAP genes exhibit isolate-specific and cross-species polymorphisms that might reflect the presence of immune pressure (35, 36, 37, 53). Moreover, antibodies produced against region II of CSP of *P. falciparum* TRAP confer some protection against the malaria parasite, identifying these proteins as potential malaria vaccine candidates (17).

The presence of TRAP family members throughout the Apicomplexa, their association with key events involved in parasite invasion and motility, and morphological and phylogenetic similarities between *C. parvum* and other apicomplexans suggest that TRAP may also be involved in the recognition and invasion of intestinal cells by *C. parvum* (22, 30). Spano et al. (49) first described and characterized a TRAP gene, TRAPC1, in *C. parvum*. TRAPC1 protein is localized to the apical end of sporozoites and is structurally related to the micronemal proteins MIC2, TRAP and Etp100, but with the notable lack of a vWA domain. The gene coding for a second TSP1 domain-containing *C. parvum* protein, TRAPC2, was partially characterized as a gene fragment (49) and contains a tandem array of TSP1 motifs. As for the TRAPs of *Plasmodium* sp. (36, 37),

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polymorphism has been observed in TRAPC1 and TRAPC2, and PCR-based polymorphism analyses of TRAPC1 and TRAPC2 have been used for genetic fingerprinting of *C. parvum* isolates of human and bovine origin (26, 33, 34, 48).

Previously, a large-scale random survey of the *C. parvum* genome conducted in our laboratory revealed the presence of multiple genomic DNA sequences similar to known apicomplexan TRAP genes, including the previously characterized TRAPC1 and TRAPC2 genes of *C. parvum* (23). Clone CpGR176 was identical to TRAPC2 at both the nucleic acid and the amino acid levels, whereas clones CpGR260 and CpGR493 were highly similar to TRAPC1 and C2 at the amino acid level (e value equals 10^{-12}). To understand the extent of expansion of possible TRAP-like proteins in the *Cryptosporidium* genome, we initiated the present study to exhaustively identify and characterize additional *C. parvum* genes containing TSP1 or vWA domains. By using apicomplexan TSP1-like domains as TBLASTN queries to search our *C. parvum* genome sequence database, we identified a total of 12 *C. parvum* genes possessing one or more TSP1-like domains. Extracellular examples of the vWA-like domain were not found in the *C. parvum* genome, either singly or in combination with TSP1 domains. The characterization of these TSP1 containing genes will not only enhance our understanding of the phylogenetic distribution and expansion of these adhesive domains within the apicomplexa (reviewed in reference 55) but also provide insights into the general mechanism employed by *C. parvum* for attachment and invasion of host cells.

MATERIALS AND METHODS

Identification of *C. parvum* TSP1 domain-containing genes. A local *C. parvum* genome database (*Crypto*DB) was constructed by using contig sequences assembled from a 9X random shotgun sequencing coverage generated by our ongoing *C. parvum* genome-sequencing project (http://www.cbc.umn.edu/ResearchProjects/AGAC/Cp/index.htm). Assembled contigs are available at http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html. To exhaustively screen for additional TSP1-like domains within *C. parvum* genes, various apicomplexan TSP1-like domains were used as TBLASTN queries of *Crypto*DB. The conservation of cysteine and tryptophan residues, and spacing of these residues, within TSP1-like domains allowed high sensitivity and high stringency in identifying putative genes. After TBLASTN searching, full-length open reading frames (ORFs) were identified within contigs of best hits by using a web-based translation program (ExPASy Translate Tool; Swiss Institute of Bioinformatics; http://www.expasy.ch/tools/dna.html). The domain structure of the resulting ORFs was determined by using reiterative PSI-BLAST (NCBI/NLM) screening of GenBank (nr database) and SMART package HMMER domain profile searches (http://www.smart.embl-heidelberg.de). Newly identified TSP1 containing genes were named to reflect, as much as possible, groupings of structural relatedness.

Isolation of total RNA from *C. parvum*-infected cells. Sterilized *C. parvum* oocysts (Iowa strain, originally from C. R. Sterling of Arizona State University) were used to infect confluent human adenocarcinoma (HCT-8; American Type Culture Collection, Manassas, Va.) cell monolayers at a concentration of one oocyst per cell, as previously described (42, 43, 58). Total RNA was prepared from mock-infected and *C. parvum*-infected HCT-8 cultures at 6, 12, 24, 48, and 72 h postinfection (p.i.) by directly lysing the cells with 4 ml of Trizol Reagent (Gibco-BRL/LifeTechnologies, Gaithersburg, Md.) according to the manufacturer's instructions. Purified RNA was resuspended in RNase-free water, and the integrity of the samples was confirmed by RNA agarose gel electrophoresis.

Expression analysis of TSP1 domain-containing genes during in vitro *C. parvum* development. To investigate differential expression of the *C. parvum* TSP1 domain genes during in vitro *C. parvum* development, sequence-specific primers (Table 1) were designed for each of the TSP1 domain genes, and a semiquantitative reverse transcription-PCR (RT-PCR) analysis was carried out as previously described (1). Briefly, 2.0 μg of total RNA was mixed with 0.74 μg of random hexamer and RNase-free water (to bring up the volume to 12 μl), heated at 70°C for 10 min, and cooled on ice. To this mixture was added a 7-μl aliquot, consisting of 4.0 μl of 5× first strand buffer, 2.0 μl of 100 mM dithiothreitol, and 1.0 μl of 10 mM deoxynucleoside triphosphate mixture. The reaction was equilibrated at 42°C for 2 min on a GeneAmp PCR system 2400 (Perkin-Elmer Cetus Corp., Norwalk, Conn.), after which 1.0 μl (200 U) of SuperScript II RTase was applied. The reaction mixture was then incubated at 42°C for 50 min, heated at 70°C for 15 min, and held at 4°C. A 2.0-μl portion of the first-strand cDNA reaction was used in a 20-μl reaction that contained 1× PCR buffer with 1.5 mM $MgCl_2$ (Perkin-Elmer, Inc.), 200 μM concentrations of each of the four deoxynucleoside triphosphates, 1.0 μM concentrations of the forward and reverse primers correspondent to each TRAP gene, 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 0.4 μCi of [^{32}P]dCTP (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). After an initial denaturation at 94°C for 2 min, the reaction mixture underwent multiple cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. The number of cycles was preoptimized (25 cycles for amplification of *Cp*TSP4 and 27 cycles for all other genes) so that accumulation of the PCR product was still in the exponential phase (data not shown). After an additional cycle of extension at 72°C for 5 min, the reactions were held at 4°C.

As the number of developing *C. parvum* life stages within infected cells changes over time, primers specific for *C. parvum* 18S rRNA (41; see Table 1) were used to normalize the amount of cDNA product of the TSP1 domain genes to that of *C. parvum* rRNA in the same sample. Since rRNA is much more abundant than any specific mRNAs, 2.0 μl of a 1:25 dilution of the cDNA samples were used and reactions underwent only 23 cycles of amplification (1). Furthermore, to monitor PCR efficiency and possible cross-contamination between reactions, a negative control with 2.0 μl of cDNA made from mock-infected HCT-8 cells and a positive control with 2.0 ng of *C. parvum* genomic DNA were also included in each batch of PCRs.

The PCR products were separated on a 4.0% nondenaturing polyacrylamide gel and signals from specific products were captured and quantified by using a phosphorimaging system (Molecular Dynamics, Inc., Sunnyvale, Calif.). The

TABLE 1. Primers used for amplification of *C. parvum* gene fragments

Primer	Nucleotide sequence	Size(s) (bp) of PCR product(s)
TRAPC1-f	GTCAGTTTGGTCAGAATGGTC	
TRAPC1-r	GTGTAGCGTCTCCAGAATCTG	395
*Cp*TSP2-f	AAGGATGCGTAGAAAGGAGAG	
*Cp*TSP2-r	TGCAAGCCTTAGTCTGAAAAA	507
*Cp*TSP3-f	TGTGTAGTTGGTCAATGGTCA	
*Cp*TSP3-r	AATTTAAGCCTGTCTTCGGAG	444
*Cp*TSP4-f	TTTTCTGAAACACACCCAGAC	
*Cp*TSP4-r	ATTTTCATCAGGGTACTTGCA	324
*Cp*TSP5-f	CTACATCAATGGCCGGATAC	
*Cp*TSP5-r	GGAAAAGTGCTTCTTTCACC	394
*Cp*TSP6-f	TAACTCATTCCCAGACAGCA	
*Cp*TSP6-r	GACACAAACCCATGTTCAGA	408
*Cp*TSP7-f	AATGGTGGTTTACCATGTCC	
*Cp*TSP7-r	CACAAGACTTGGAGCAAACA	400
*Cp*TSP8-f	GTACTTGCAATGGTGGAACA	
*Cp*TSP8-r	TGATTAGATGACGGACATGC	409
*Cp*TSP9-f	CTCTGCCGGTGGTAGTATTT	
*Cp*TSP9-r	GGACCAAGAACTCCATTCTG	391
*Cp*TSP10-f	GTCTGCATGCTCAGACACAT	
*Cp*TSP10-r	CACAATCAGTTGGAACAGGA	401
*Cp*TSP11-f	GGGAAGCCCTATGATACTGA	
*Cp*TSP11-r	TGTACACCCCTGAGTTTGAA	412
*Cp*TSP12-f	CAGTATCTGCAAATGGGTCA	
*Cp*TSP12-r	CATGGACCTAATTGGCAAC	403
*Cp*TSP8 intron-f[a]	TCCTTGTCATAGCATAGTGT	
*Cp*TSP8 intron-r[a]	GACCATAATGACTCTCTTA	790[b], 564[c]
18S rRNA-f[d]	CTCCACCAACTAAGAACGCC	
18S rRNA-r[d]	AGAGATTGGAGGTTGTTCCT	212

[a] Primers spanning the region that contains the 226-bp predicted intron.
[b] Expected length of the unspliced genomic DNA PCR product.
[c] Expected length of the cDNA PCR product.
[d] Primers described by Rochelle et al. (41).

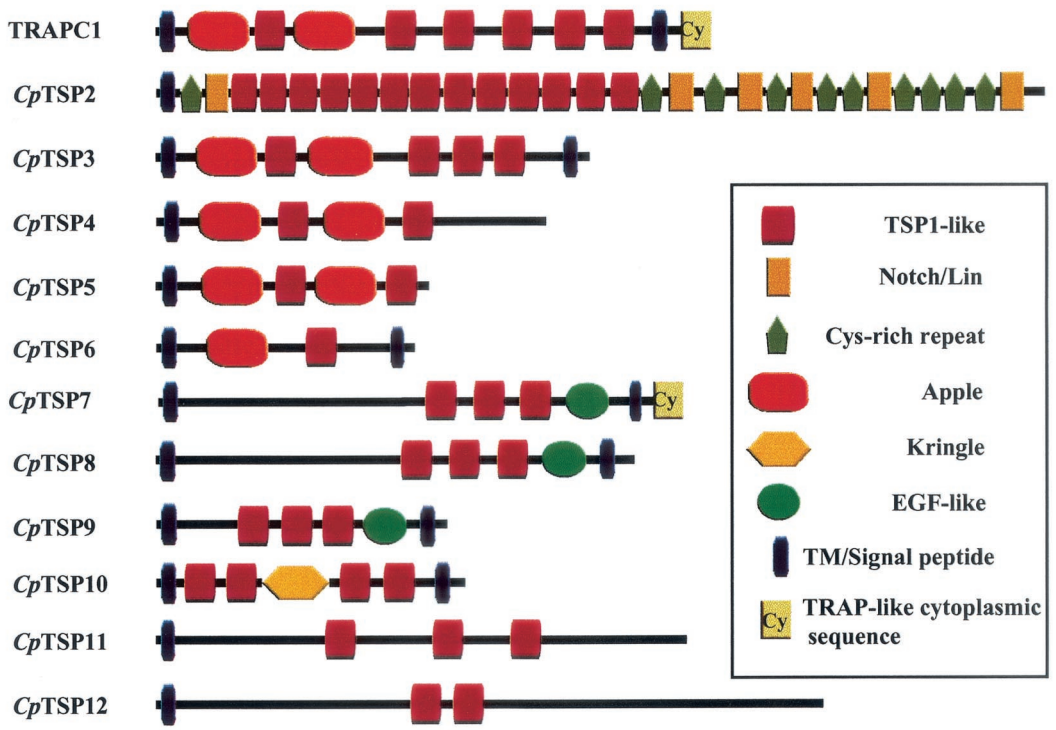


FIG. 1. Schematic of *C. parvum* TSP1 domain-containing genes.

expression level of TSP1 domain genes at each time point was calculated as the ratio of its RT-PCR product signal to that of the *C. parvum* 18S rRNA (1). Three independent time course experiments were used in the analysis. Each of the RNA samples from *C. parvum*-infected HCT-8 cells was demonstrated to be free of contaminating *C. parvum* genomic DNA by the lack of amplification product from a sample not subjected to RT (data not shown). The mean expression level of each of the TSP1 domain genes was calculated, and the standard deviation was determined. The percentage of expression of each TSP1 domain gene at each time point was also reported as a percentage of the highest level of expression.

RESULTS

***C. parvum* genome possesses a superfamily of TSP1-like domain genes.** In the present study a local *Cryptosporidium* genome database (*Crypto*DB) was constructed and screened via standalone TBLASTN by using various apicomplexan TSP1 and vWA domains as queries. TSP1-like domains are excellent queries by virtue of two conserved tryptophan residues and either four or six conserved cysteine residues. An exhaustive screening process, in which each newly identified gene was in turn used as a TBLASTN query of *Crypto*DB, revealed the presence of TSP1-like domains within 12 *C. parvum* genes. TSP1 domains are therefore greatly expanded within the *C. parvum* genome. In contrast, vWA domains were not identified within any of the 12 TSP1 domain-containing genes, as assessed by PSI-BLAST screening of GenBank (nr) by using each gene, and fragments thereof, as queries. Additionally, vWA domains were not identified after screening of each TSP1 domain-containing gene by using an extracellular vWA HMMER profile (SMART database; http://smart.embl-heidelberg.de). Further extensive screening of *Crypto*DB by using as queries all known apicomplexan and numerous vertebrate vWA domains did not identify a vWA domain, either singly or in combination with TSP1 domains. We therefore propose that *Cryptosporidium* does not utilize vWA in extracellular adhesive interactions and that a capacity for gliding motility and invasion does not require this adhesive module.

Cryptosporidium TSP1 domains were previously described in TRAPC1 (49) and within two gene fragments, TRAPC2 (50) and TRAPC3 (49; GenBank accession number AF073838). Genome analysis in the present study revealed that TRAPC2 and TRAPC3 are fragments of a single ORF and are herein named *Cp*TSP2. This departure from the previous *Cryptosporidium* TRAP nomenclature reflects the observation that, unlike TRAPC1, *Cp*TSP2 does not possess "TRAP/MIC2"-like motifs, namely, a transmembrane region combined with a short, acidic cytoplasmic domain having a conserved carboxy-terminal proximal tryptophan residue. The newly identified TSP1-like domain-containing genes are similarly herein termed *Cp*TSP3 through *Cp*TSP12. TSP1 domains are present as multiple copies within all *C. parvum* TSP1 domain genes, except for a single domain within *Cp*TSP6, and are notably present as 13 tandemly arrayed copies within *Cp*TSP2 (see schematic, Fig. 1). The *Cp*TSP2 gene encodes a large, 3,530-amino-acid protein that has a unique multidomain structure additionally composed of multiple Notch/Lin-like domains and a novel cysteine-rich domain that is repeated 10 times (alignments shown in Fig. 2).

The domain structure of the *C. parvum* TSP1 domain genes was analyzed by using the web-based SMART package (http://smart.embl-heidelberg.de) of HMMER domain consensus profiles. Domains were additionally identified or confirmed via

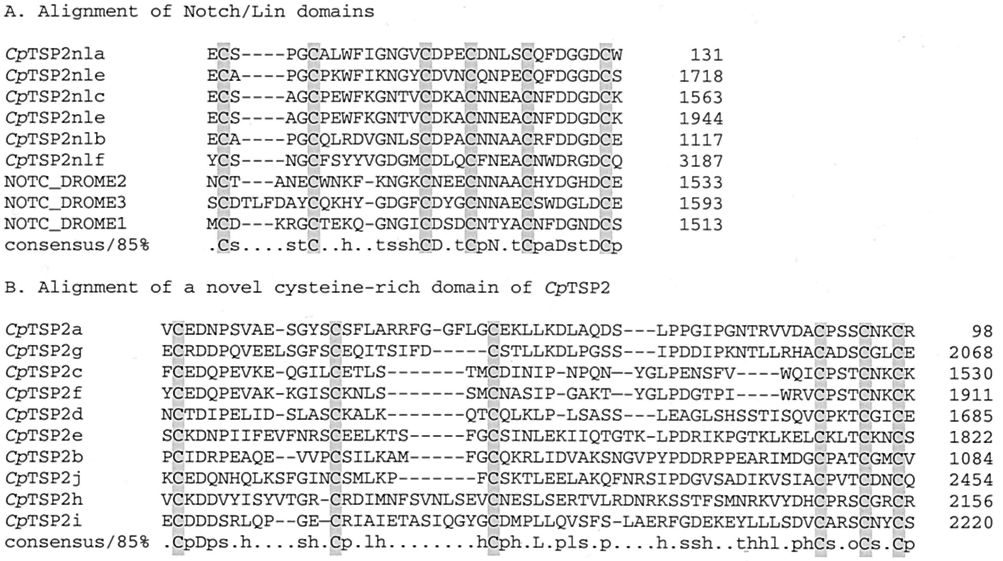


FIG. 2. Alignment of Notch/Lin-like domains (A) and a novel adjacent repeated domain (B) from *C. parvum Cp*TSP2. Conserved cysteine residues are shaded (NOTC_DROME; GenBank accession number P07207).

reiterative PSI-BLAST analysis of GenBank (nr database) by using TSP1 domain genes, and fragments thereof, as queries. In this manner additional domains were identified and the domain structure was addressed for all 12 *Cryptosporidium* TSP1 domain genes (Fig. 1). All TSP1 domain genes possess a putative signal peptide sequence near the amino terminus. *Cp*TSP3 and *Cp*TSP6 through *Cp*TSP10, as well as the previously described TRAPC1, have putative transmembrane regions near their carboxy termini. *Cp*TSP8 and *Cp*TSP10 have short cytoplasmic domains less than 10 amino acids in length, whereas the other transmembrane proteins have cytoplasmic domains of greater than 40 amino acids. The latter proteins possess tyrosine-rich motifs within their cytoplasmic tails that might represent recently described microneme or rhoptry targeting sequences (14, 21) and warrant further study to address protein localization. The cytoplasmic tail of *Cp*TSP7 has an acidic nature and tryptophan residue near the -COOH terminus reminiscent of *Plasmodium* TRAP and *Toxoplasma* MIC2. *Cp*TSP7 is thus a candidate for a "TRAP family" gene and is therefore potentially involved in extracellular adhesive events coupled with cytoplasmic interaction, either directly or indirectly, with an actin/myosin motility apparatus.

TRAPC1 and *Cp*TSP3 are located on opposite strands within the same genomic contig and likely have a paralogous relationship via gene duplication. Similarities in domain structure with *Cp*TSP4 through *Cp*TSP6 suggests that they might also have an ancestral relationship via gene duplication.

TRAPC1 and *Cp*TSP3 through *Cp*TSP6 contain divergent Apple domains. TRAPC1 and *Cp*TSP3 through *Cp*TSP5 share an amino-terminal region architecture consisting of two Apple domains intervened with a single TSP1-like domain. The second Apple domain is followed by a variable number of additional TSP1-like domains. Apple domains were initially described in the human kallikrein gene (9) and have likely arisen in Apicomplexa via lateral capture, present in *Toxoplasma* (3) and *Eimeria* (4). Their presence in *Cryptosporidium* suggests that, like TSP1 domains, their lateral transfer preceded species divergence of the apicomplexan species. Alignment of *C. parvum* Apple domains (Fig. 3) delineates two motifs differing by a pair of cysteines, presumably participating in an additional disulfide bond. Although Apple domains containing the extra pair of cysteines are not readily identified by PSI-BLAST or SMART analysis, BLAST screening of *Crypto*DB with kallikrein and other Apple domain-containing queries identifies the putative Apple domains in *Cp*TSP4 and *Cp*TSP6. Alignments show that this domain is repeated twice in TRAPC1 and *Cp*TSP3 through *Cp*TSP5 and present as a single copy in *Cp*TSP6. Based upon the conservation of structure and repetition within TSP1 domain genes, we propose that this is a discrete globular domain and is probably a divergent Apple domain.

Introns within *Cp*TSP8 and *Cp*TSP9. *Cp*TSP7, *Cp*TSP8, and *Cp*TSP9 share a domain structure composed of three contiguous TSP1-like domains, followed by a single epidermal growth factor-like domain adjacent a putative transmembrane domain. The domain structure and conservation of cysteine residue spacing suggests that they also have a parologous relationship arising via gene duplication. Comparison of the *Cp*TSP8 ORF with *Crytosporidium* EST databases (http://www.ebi.ac.uk/parasites/cparvEST.html) revealed the presence of a 266-bp intron (nucleotide positions 961 to 1186 relative to the start codon; see schematic in Fig. 4). Comparison of the domain structure between *Cp*TSP8 and *Cp*TSP9 identified a 77-bp intron (nucleotide positions 132 to 208) with the *Cp*TSP9 ORF. The presence of an intron has only one precedent within *Cryptosporidium*, the beta-tubulin gene (5). Functional splicing of the *Cp*TSP8 (Fig. 4) and *Cp*TSP9 (data not shown) introns were confirmed by comparison of PCR product lengths from genomic DNA versus reverse-transcribed RNA templates (Fig. 4A) and sequencing of the resulting PCR product derived from cDNA (Fig. 4B). *Cp*TSP8 and *Cp*TSP9 are located on the same contig on opposite strands, supporting a paralogous relation-

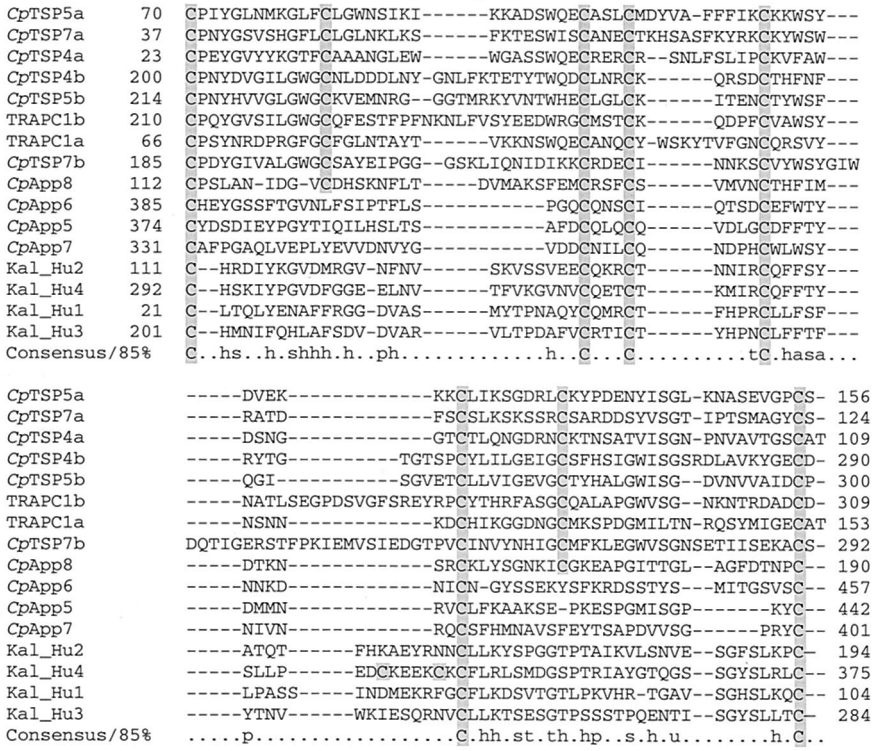


FIG. 3. Alignment of Apple-like domains from *C. parvum* genes with Apple domains from human kallikrein (GenBank accession number P03952.) *Cp*App5 through *Cp*App8 are Apple domain-containing genes within CryptoDB identified via TBLASTN analysis and have not been previously described. Cysteine residues are shaded.

ship. *Cp*TSP7 and *Cp*TSP8 both contain amino-terminal regions of roughly 40 kDa which do not have any similarity to each other nor to any gene in GenBank as queried by using PSI-BLAST.

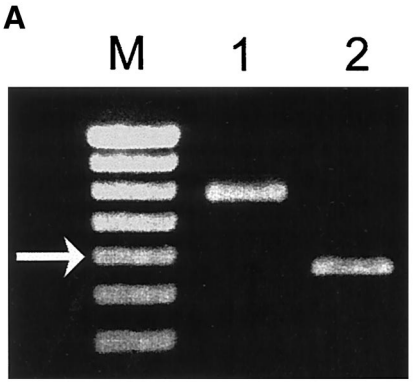
Additional *C. parvum* TSP1 domain genes. *Cp*TSP10 through *Cp*TSP12 show no relation in their domain superstructure, either to each other or to the other TSP1 domain genes and yield no clues that they may have arisen via recent gene duplication. *Cp*TSP10 is notable for the presence of a kringle domain, whereas *Cp*TSP11 and *Cp*TSP12 do not contain recognizable modules other than TSP1-like domains. Kringle domains are another example of lateral capture of an extracellular protein module and have not been previously described within apicomplexan genes. Complete annotation of the *Plasmodium* and *Toxoplasma* genomes will give an indication of the extent of expansion of this domain in Apicomplexa. Preliminary BLAST screens of the *Crypto*DB did not identify additional genes possessing kringle domains. The validity of the kringle domain in *Cp*TSP10 was demonstrated first by its identification with the SMART package of HMMER consensus profiles (e value equals 10^{-7}), and the fact that the *C. parvum* kringle domain readily identifies plasminogen activator and other kringle domain-containing genes after a PSI-BLAST screen of GenBank (e value equals 10^{-6}).

Expression analysis of TSP1 domain genes. RT-PCR analysis of gene expression of TRAPC1 and *Cp*TSP2 thru *Cp*TSP12 is presented in Fig. 5 and 6. As the number of developing *C. parvum* life stages within infected cells changes over time, primers specific for *C. parvum* 18S rRNA (41; see Table 1) were used to normalize the amount of cDNA product of the TSP1 domain genes to that of *C. parvum* rRNA in the same sample. The mRNA level of TRAPC1 in infected cultures display a high degree of variability, demonstrating that TRAPC1 expression is clearly regulated as *C. parvum* progresses through its life cycle. The mRNA levels of *Cp*TSP2 thru *Cp*TSP12 in infected HCT-8 cells are also illustrated in Fig. 6, and the percent expression of TSP1 domain genes in infected HCT-8 cells at each sampled time point is presented in Table 2.

As illustrated in Fig. 6, expression of the TSP1 domain genes is regulated in a complex fashion. Some of TSP1 domain genes are expressed at high levels at both early and late stages of infection (TRAPC1, *Cp*TSP3, and *Cp*TSP11), whereas others are expressed more abundantly at late stages (*Cp*TSP2, *Cp*TSP5, *Cp*TSP6, *Cp*TSP8, and *Cp*TSP9) or only at early stages of infection (*Cp*TSP4).

DISCUSSION

Accumulating evidence suggests that the molecular machinery for gliding motility and cell invasion is conserved across apicomplexan genera (22). TRAP homologues in different apicomplexans share structural and functional domains, indicating that they constitute a family of functionally homologous proteins playing key roles in the ability of the parasites to recognize and invade host cells. TRAP proteins localize to apical region microneme secretory vesicles and are released via calcium-mediated signals to the cell surface at the apical pole



B

ACCTTTCAAT↓GTAAGTTTACTTATGA
TTATTAAGATAATAAGCCTATTTATTG
AGGTAGGGAGAGAGAGAGAGAGAGA
GAGAGAGAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGAGAGAGAGA
GGAAGAAAAAGGAAGAAGCATGATA
GGCTTGGAAAATGATCTTAATAAGAA
TTTTAACATTTCAAAATTGATTAACTT
TTTATATTTTTTCTTCTCTCATCTTTAT
AG↓TCGATATTGA

FIG. 4. PCR analysis and sequencing of the *C. parvum* *Cp*TSP8 region that contains a predicted intron. (A) *C. parvum* genomic DNA templates (lane 1) and reverse-transcribed RNA of *C. parvum*-infected HCT-8 cells (48 h p.i.; lane 2) were amplified by using sequence-specific primers spanning the 226-bp intron (Table 1), and the resulting PCR products were separated on 1.0% agarose gel. M, 100-bp DNA ladder. The arrowed band is 600 bp. (B) The RT-PCR product illustrated in lane 2 of panel A was TA cloned, and the insert of a positive colony was sequenced from both directions by using PCR primers. The functional splicing sites are indicated by arrows. Also included are 10 nucleotides of the exon flanking each side of the intron.

(30, 55). A capacity for gliding motility is proposed to involve extracellular adhesion to host tissue substrates, coupled across the transmembrane region to cytoplasmic interaction with a subpellicular myosin/actin microfilamentous motor apparatus. In this fashion, adhesive receptors stream from the apical to posterior poles of the parasite surface, propelling the parasite forward either on host cell surfaces or during invasion of host cells. Cell invasion is thought to involve additional formation of a tight junction forming between the parasite and host membranes, with anterior-to-posterior migration of this junction driven by the subpellicular motor filaments (15, 16).

The TRAPC1 gene isolated by Spano et al. (49) encodes a protein architecturally similar to *Toxoplasma* MIC2, with the notable absence of a vWA-like domain. Despite the lack of a vWA module, TRAPC1 is a prominent candidate for a functional *Cryptosporidium* homologue of MIC2 by virtue of possessing a signal peptide sequence; multiple tandemly arrayed TSP1 domains; a transmembrane region; and a characteristic short, acidic cytoplasmic domain containing a carboxy-terminal tryptophan residue conserved in TRAP, CTRP, MIC2, and Etp100. In addition, TRAPC1 protein is localized within the apical complex of excysted *C. parvum* sporozoites, similar to the micronemal destination of TRAP, CTRP, MIC2, and Etp100. TRAPC1 is herein described to possess Apple domains and thereby is further distinguished from *Plasmodium* TRAP and *Toxoplasma* MIC2, which lack Apple domains. In the present study the identification of a superfamily of *C. parvum* TSP1 domain-containing genes adds new members to the growing family of TRAP-like proteins (55) and provides information on the phylogenetic distribution of this protein family in the *C. parvum* genome. Of these genes only *Cp*TSP7 shares with TRAPC1 and other apicomplexan TRAP family members a transmembrane domain followed by a short, acidic cytoplasmic domain and a conserved COOH-terminal tryptophan residue. Although the functional roles of TRAPC1 and *Cp*TSP2 through *Cp*TSP12 have yet to be elucidated, the similar invasion machinery of *C. parvum* to that of other apicomplexans suggest that *C. parvum* TSP1 domain containing genes may also be involved in the invasion process of host intestinal cells by *C. parvum* zoites.

An interesting result of this *Cryptosporidium* genome-wide screen is the failure to identify a vWA domain, either alone or in tandem with a TSP1 domain. All known apicomplexan vWA domains were tried as TBLASTN queries, as well as various vWA domains of higher eukaryotes—all without success. Although it is possible that *Cryptosporidium* possesses vWA domains that have diverged beyond recognition by TBLASTN screens, it is unlikely that any vWA domain would be missed by SMART profile screening or reiterative PSI-BLAST analysis if it were to reside within any of the 12 identified TSP1-containing genes. Because TSP1 domains are superb TBLASTN queries, and it is unlikely that TSP1 domains were overlooked in the present study, it is therefore assumed that no gene exists in the current *Cryptosporidium* genome databases possessing both a TSP1 domain and a vWA domain. Thus, *Cryptosporidium* is fundamentally distinct in this regard in comparison with all apicomplexans analyzed to date: *Plasmodium*, *Toxoplasma*, *Eimeria*, and *Neospora*. Whether vestigial vWA domains were lost in *Cryptosporidium* or acquired in other apicomplexans

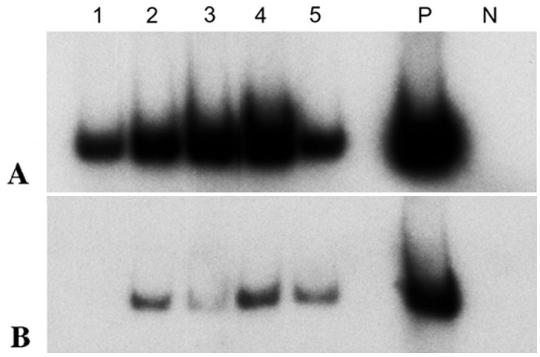


FIG. 5. RT-PCR analysis of *C. parvum* TRAPC1 expression during in vitro development in HCT-8 cells. (A) Total RNA (2.0 μg) isolated from *C. parvum*-infected HCT-8 cells at 6, 12, 24, 48, and 72 h p.i. (lanes 1 to 5, respectively) was reverse transcribed by using random primers, and 2.0 μl of a 1:50 dilution of each cDNA reaction was subjected to 23 cycles of amplification in the presence of [^{32}P]dCTP and primers specific for *C. parvum* 18S rRNA (Table 1). P, positive control (*C. parvum* genomic DNA used as a template in PCR); N, negative control (total RNA from mock-infected cells used in RT). (B) Two microliters of each cDNA reaction was amplified by using primers specific for *C. parvum* TRAPC1 (Table 1).

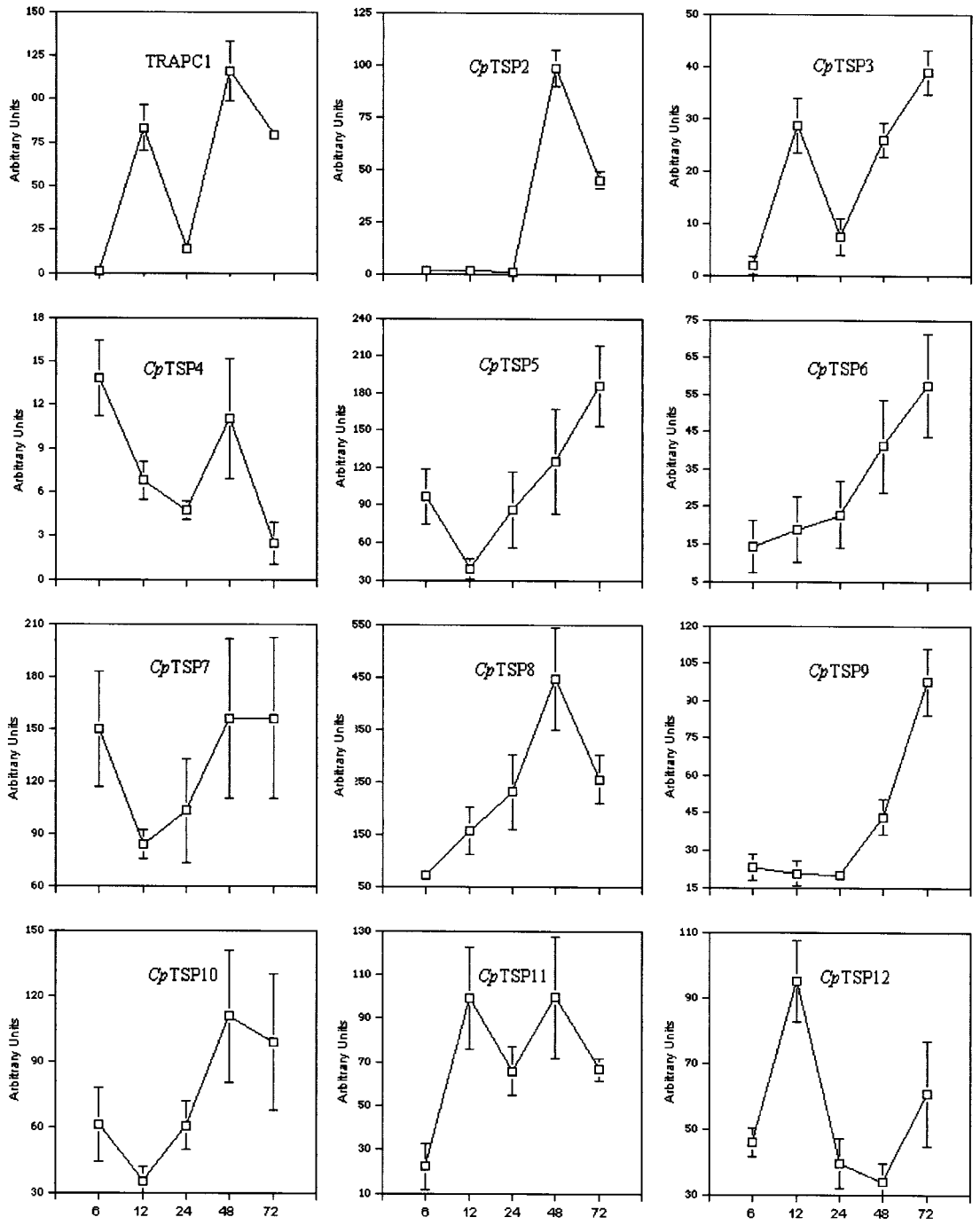


FIG. 6. Expression profiles of *C. parvum* TRAPC1 and *Cp*TSP2 to *Cp*TSP12 during in vitro *C. parvum* development in HCT-8 cells. The data are expressed as the ratio of TRAPC1 and *Cp*TSP2 thru *Cp*TSP12 signal to *C. parvum* 18S rRNA signal present in each of the infected samples, as determined by using a phosphorimaging system. Error bars indicate the standard deviation of the mean of three independent experiments.

after the split into species is unknown. In *Plasmodium* spp., an intact vWA domain is essential for TRAP-mediated invasion of mosquito salivary glands but is not required for gliding motility (25, 59). A simple model would propose that vWA domains are involved solely in the insect stage of parasite life cycles, but *Toxoplasma* MIC2 presumably requires a vWA domain whereas the *Toxoplasma* parasite does not utilize an insect vector. Regardless of whether vWA domains never entered the *Cryptosporidum* genome or became vestigial and lost, it can be proposed that, if *Cryptosporidium* TSP superfamily proteins are involved in the motility and invasion of intestinal epithelial cells, then vWA domains are not essential for this process.

Due to its critical role in parasite motility and attachment to

TABLE 2. Expression of *C. parvum* TSP1 domain-containing genes during development in HCT-8 cells

Gene	Relative expression (%) at different time points				
	6 h	12 h	24 h	48 h	72 h
TRAPC1	1	72	12	100	68
*Cp*TSP2	2	1	1	100	46
*Cp*TSP3	5	74	19	67	100
*Cp*TSP4	100	49	34	80	18
*Cp*TSP5	52	21	53	67	100
*Cp*TSP6	25	33	40	72	100
*Cp*TSP7	96	54	66	100	100
*Cp*TSP8	16	35	52	100	57
*Cp*TSP9	24	21	20	44	100
*Cp*TSP10	55	30	55	100	90
*Cp*TSP11	22	100	66	100	67
*Cp*TSP12	48	100	43	36	64

host cells, *P. falciparum* TRAP has been suggested as a candidate vaccine gene, and it was shown that the disruption of TRAP dramatically blocked parasite motility and resulted in much-reduced infectivity (17, 44). Previously, mouse monoclonal antibodies to *C. parvum* TRAPC1 functional domains were used in an in vitro inhibition assay to assess their abilities to inhibit parasite infection and growth in host cells. However, only limited reductions on the number of *C. parvum* development stages were observed at 48 h p.i. (6). Since it is now clear that that there are several additional TSP1 domain genes and that at any stage of infection process there is one or more TSP1 domain genes expressed at high levels (Fig. 6), antibodies against one TSP containing protein may not be sufficient to dramatically inhibit *C. parvum*'s ability to invade host cells. Alternatively, TSP1 domain genes may represent only one of the multiple invasion pathways used by *C. parvum*.

Previous studies found that TRAP homologues from different *Plasmodium* species were expressed exclusively at the sporozoite developmental stage and that the expression pattern of *P. falciparum* TRAP parallels the development of *P. falciparum* infectivity during sporozoite ontogeny (11, 40). In the present study, it was found that *C. parvum* TSP1 domain gene homologues display distinct expression profiles; some of them are expressed highly at early stages of infection (*Cp*TSP4, *Cp*TSP7, and *Cp*TSP11), whereas some others are expressed mostly at late stages. The overall expression of all TRAPs peaks at 48 h p.i., although expression of specific TSP1 domain genes is higher at earlier stages than at later stages (i.e., *Cp*TSP2 and *Cp*TSP4). However, since *C. parvum* has a multistage life cycle and the in vitro development is not synchronous, it remains to be elucidated whether the expression of specific TSP1 domain genes is restricted to a specific developmental stage.

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