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Vital functions of the malarial ookinete protein CTRP reside in the A domains

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Abstract

The transformation of malaria ookinetes into oocysts occurs in the mosquito midgut and is a major bottleneck for parasite transmission. The secreted ookinete surface protein CTRP (circumsporozoite- and TRAP-related protein) is essential for this transition, and hence constitutes a potential target for malaria transmission blockade. CTRP is a modular multidomain protein containing six tandem von Willebrand factor A-like (A) domains and seven tandem thrombospondin-like (TS) domains. Here, we present the first structure-function analysis of CTRP using genetically modified *Plasmodium berghei* parasites expressing mutant versions of the *ctrp* gene. Our data show that the A domains of CTRP are critical for ookinete gliding motility and oocyst formation whilst unexpectedly, its TS domains are fully redundant. These results may have important implications for the design of CTRP-based transmission blocking strategies.

Keywords

CTRP; Transmission; Motility; Invasion; *P. berghei*; *Anopheles*

1. Introduction

When a female mosquito takes up a bloodmeal from a malaria infected host, gametocytes undergo gametogenesis. After fertilization, the zygote escapes the hostile gut environment, where it is exposed to mosquito digestive enzymes and the immune system of the vertebrate host, by transforming into a motile ookinete which invades the midgut epithelium. Having passed through the epithelial cell layer, the ookinete resides between the epithelia and the basal lamina and develops into an oocyst. The oocyst undergoes growth and asexual replication to form sporozoites which upon release travel through the haemocoel to the salivary glands where they await a delivery to the next vertebrate host.

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Invasion of the midgut epithelium by the ookinete requires motility, adhesion and subsequent disruption of the host cell. The adhesive surface protein circumsporozoite- and TRAP-related protein CTRP (Trottein et al., 1995) is essential to motility and infectivity (Dessens et al., 1999; Yuda et al., 1999a; Templeton et al., 2000) and has been shown to bind to the basal lamina components collagen (Arrighi and Hurd, 2002) and laminin (Arrighi and Hurd, 2002; Mahairaki et al., 2005). In *P. berghei*, the protein is first produced approximately 10 hrs after gametocyte activation (Dessens et al., 1999; Yuda et al., 1999b) and is secreted from the micronemes (Dessens et al., 1999; Limviroj et al., 2002; Kaneko et al., 2006). The 215 kDa protein belongs to the thrombospondin-related adhesive protein (TRAP) family (Naitza et al., 1998; Tomley and Soldati, 2001, Fig. 1), members of which share common features: a signal peptide, thrombospondin type 1 repeat-like (TS) domains, a transmembrane (TM) domain and a short cytoplasmic tail. CTRP contains seven TS domains of which three are conserved and four degenerate (Trottein et al., 1995) and which are homologous to TS domains from *Plasmodium* TRAP and other apicomplexan TRAP family proteins, circumsporozoite protein (CSP), vertebrate thrombospondins, F-spondin and properdin (Trottein et al., 1995; reviewed by Naitza et al., 1998). TS domains have been shown to bind to extracellular matrix components such as fibronectin, laminin and collagen, to fibrinogen and glycosaminoglycans (GAGs) such as heparin, heparin sulphate and chondroitin sulphate (Naitza et al., 1998).

CTRP also contains six copies of domains related to von Willebrand factor A domains (here referred to as A domains), four of which are conserved within the molecule and between species (Trottein et al., 1995; Kaneko et al., 2006), and contain metal ion-dependent adhesion sites (MIDAS). The A domains of CTRP show homology to A domains from other apicomplexan TRAP family proteins as well as to an inserted sequence of some integrins and A domains from von Willebrand factor, certain complement factors and extracellular matrix proteins (Trottein et al., 1995). A domains are known to bind platelet glycoprotein Ib, factor X, fibrinogen and inter-cellular adhesion molecules (ICAMs), collagens, laminin, heparin and complement components (reviewed by Colombatti and Bonaldo, 1991). Whilst multiple copies of the TS domains are common in this protein family, CTRP is unique in that it also contains multiple A domains (Fig. 1). The only exception to this is TRAP-like protein (TLP), which contains a second A domain, albeit poorly conserved (Moreira et al., 2008).

In the *Plasmodium* sporozoite protein TRAP, both the A domain and the TS domain have been shown to play roles in infectivity (Wengelnik et al., 1999; Matuschewski et al., 2002). Deleting 15 amino acids of the TS domain (WSPCSVTCGKGTRSR in *P. falciparum* TRAP) abolished motility (Wengelnik et al., 1999). Further TRAP mutants with mutations in the A or separately, in the TS domains were shown to have no influence on *P. berghei* sporozoite gliding motility, but nevertheless impaired sporozoite infectivity of both salivary glands and hepatocytes (Matuschewski et al., 2002). The A domain of TRAP binds heparin and the human hepatoma cell line HepG2, probably using heparan sulphate as a ligand (McCormick et al., 1999) and has been suggested to bind saglin on the mosquito salivary gland (Ghosh et al., 2009), a protein previously not demonstrated to be modified with GAGs (Okulate et al., 2007). The TS domain of TRAP binds to sulphated GAGs possibly present

on salivary glands (Dinglasan, personal communication) and HepG2 cells (Müller et al., 1993) and is important for salivary gland invasion and is involved in establishing a liver infection (Matuschewski et al., 2002).

Functionally, CTRP and TRAP appear to play equivalent roles in the ookinete and in the sporozoite respectively: both are essential for invasion (Dessens et al., 1999; Yuda et al., 1999a; Templeton et al., 2000; Matuschewski et al., 2002) and motility (Sultan et al., 1997; Dessens et al., 1999; Matuschewski et al., 2002). However, whilst the ookinete only traverses midgut epithelial cells without forming a parasitophorous vacuole (PV), the sporozoite traverses cells in the salivary gland and in the vertebrate host before it invades the final hepatocyte forming a PV. This may be why the sporozoite contains two other TRAP family proteins: TLP (Heiss et al., 2008; Moreira et al., 2008) and TRAP-related protein (TREP, Combe et al., 2009). Whilst TLP is thought to be involved in cell-traversal in the vertebrate host (Moreira et al., 2008), TREP has been shown to be important for gliding motility of haemolymph sporozoites and invasion of salivary glands (Combe et al., 2009).

The model for apicomplexan movement postulating an actin-dependent molecular motor was first described in *Eimeria magna* using cytochalasin B to inhibit actin polymer formation (Jensen and Edgar, 1976). Studies with *Eimeria* sporozoites (Dubremetz, 1978; Russell and Sinden, 1981) and *Gregarina* (King, 1981) led to the “capping” model of motility where parasite surface molecules were implicated to adhere to the substratum and translocated to the posterior pole of the parasite while the parasite moves forward (Russell and Sinden, 1981; Russell, 1983). TRAP was implicated to be this surface molecule in *Plasmodium* sporozoites by inhibition studies of HepG2 invasion and of sporozoite motility with anti-TRAP antibodies (Müller et al., 1993; Spaccapelo et al., 1997) and was shown to be necessary for sporozoite gliding motility using genetic knockout studies (Sultan et al., 1997; Matuschewski et al., 2002). TRAP family proteins are thought to be secreted to the parasite surface, translocated from the anterior to the posterior (Sibley, 2004) and finally proteolytically released by a rhomboid protease (Brossier et al., 2003; Soldati et al., 2004). CTRP has a predicted rhomboid cleavage site (Dowse and Soldati, 2005) and is reportedly processed by the rhomboid protein ROM4 (Baker et al., 2006). The cleavage of CTRP is supported by the observation that motile ookinetes shed a trail of CTRP (Lecona et al., 2010), similarly to the way by which sporozoites shed trails of TRAP (Kappe et al., 1999) and CSP (Stewart and Vanderberg, 1992).

TRAP and CTRP interact with aldolase mediated by a conserved tryptophan in their cytoplasmic tails (Buscaglia et al., 2003; Jewett and Sibley, 2003; Heiss et al., 2008). The resulting complex binds actin (Buscaglia et al., 2003; Jewett and Sibley, 2003) and is indirectly linked to the inner membrane complex (reviewed by Keeley and Soldati, 2004; Baum et al., 2006; Soldati-Favre, 2008; Daher and Soldati-Favre, 2009), possibly mediated by members of the GAPM (glideosome-associated protein with multiple-membrane spans) family (Bullen et al., 2009).

Using CTRP knock-out parasite lines in *P. berghei* (Dessens et al., 1999; Yuda et al., 1999a) and in *P. falciparum* (Templeton et al., 2000) revealed the protein's importance in invasion and motility. In contrast to TRAP, the individual roles of the A and the TS domains have not

been elucidated. Here, we present the first structure/function analysis of CTRP by generating genetically modified *P. berghei* parasites expressing mutant *ctrp* lacking either all the A domains, or all the TS domains. We show that ookinetes expressing mutant *ctrp* containing all the A domains, but no TS domains, develop efficiently into oocysts in vector mosquitoes. In contrast, no oocysts are formed if ookinetes express *ctrp* lacking all the A domains, despite the presence of all the TS domains. We further show that the presence of the A domains of CTRP is sufficient to render ookinetes fully motile, whereas ookinetes lacking the CTRP A domains display highly reduced motility, fail to associate with the midgut *in vivo* and are incapable of invading the midgut epithelium.

2. Materials and methods

All experiments involving laboratory animals were performed in accordance with the EU regulations 'EU Directive 86/609/EEC' and the regulations of the United Kingdom Animals (Scientific Procedures) Act 1986.

2.1 Parasite maintenance, culturing and purification

Parasites were maintained as cryopreserved stabulates and by blood passage in Tuck-Ordinary mice (Harlan Laboratories). To harvest blood stage parasites, mice were anaesthetised with xylazine/ketamine hydrochloride/PBS 1:2:3. Schizonts were cultured using standard protocols (Janse et al., 2006b). To obtain ookinetes, gametocyte-enriched mouse blood was diluted 1:40 in ookinete medium (Moon et al., 2009)/20% FBS and cultured as previously described (Sinden, 2002).

2.2 Generation of transgenic parasites

A 682 bp fragment encompassing the 3' UTR and the 3' region of *ctrp* was amplified from *P. berghei* genomic DNA using the primers 9-CTMCT3'/U (5'-GGTACCGGTAGTAGTCAGGAAATCAAATGG-3') and 10-CTMCT3'/D (5'-ACGCGTCGAATAATGGTGAAAATAGTGAAG-3') and excised from an intermediate vector using *MluI* and *KpnI*. The signal sequence of *ctrp* together with its 5' UTR was amplified as a 2158 bp fragment using 3-CPROSIG/U (5'-ACGCGTGCCTTCTTGATATATTTTTTTGAAG-3') and 4-CPROSIG/D (5'-GAATTCGTGAAAATATGTGTATTTTTCTTAAATTTG-3') and was excised from an intermediate vector with *EcoRI* and *MluI*. Both fragments were ligated into *EcoRI-KpnI*-digested pD_b-D_h^Δ D_b (de Koning-Ward et al., 1998), resulting in pCN-CTRP. A 949 bp fragment of the *ctrp* 3' UTR was amplified with the primers 17-C3'/U (5'-GGCCGTTGACTGAGGTAAAGGCATCCCTT-3') and 18C3'/D (5'-GGCCGTTGACGAATGCTCATATGCGTGTG-3'), digested with *HincII* and cloned into *HincII*-digested pCN-CTRP to obtain pCN-CTRP/DHR. The 6 A domains were amplified with 5-CVWA/U (5'-GCGCGCCACAAATAAATTTATTATGTATTTCTACAGAAGA-3') and 6-CVWA/D (5'-ACGCGTCACCATGTGCAGGT-3'), digested with *MluI* and *BssHII* and cloned into *MluI*-digested pCN-CTRP/DHR to give pCAN-CTRP/DHR. All 7 TS domains were amplified with 7-CTSP/U (5'-ACGCGTCTAAACGGGAGGTACACAAG-3') and 8-CTSP/D (5'-GCGCGCACCCCGAAGATGCAGAAT-3'), digested with *MluI* and *BssHII* and cloned

into *Mlu*I-digested pCN-CTRP to give pCTSN-CTRP. The 3'-UTR of *ctrp* was then cloned into *Hinc*II-digested pCTSN-CTRP to obtain pCTSN-CTRP/DHR. The plasmid backbone was excised with *Nhe*I and *Sbf*I and 1-5 µg of digested plasmid were transfected into purified *P. berghei* ANKA clone 234 or 507c11 (Janse et al., 2006a) schizonts using the Human T Cell Nucleofector Kit (Amaya, Janse et al., 2006a). After two rounds of drug selection in mice using 70 mg/l pyrimethamine in drinking water, the transgenic lines were cloned by limiting dilution.

2.3 Southern blotting and diagnostic PCR

Genomic DNA was extracted from mixed, purified *Plasmodium* blood stages using the Wizard Genomic DNA Purification Kit (Promega). For Southern blotting, the DNA was digested with *Bst*BI and separated on 0.8% agarose gels. The DNA was then transferred to Hybond-N+ (GE Healthcare) nylon membrane and hybridised to the $\alpha^{32}\text{P}$ -dATP-labelled probe using the High Prime DNA Labelling Kit (Roche) at 68 °C using standard procedures (Sambrook, 1989). For diagnostic PCR, 3-DHR/U (5'-CCAAATGATTGTGGTTGGATG-3') was used in combination with 2-DHR/D (5'-CAGTTTAGCGAACCAACCATG-3') to detect the integrated plasmid in the transgenic lines and with CT/D (5'-GAAGAAAACGAAGATTTCGAAG-3') to detect bands in the background as well as the transgenic lines. Primers amplifying the unrelated gene guanylyl cyclase β were used as a positive control.

2.4 RT-PCR analysis

Ookinetes were cultured, enriched by lysing the red blood cells in 150 mM NH_4Cl , 10 mM KHCO_3 , 1 mM EDTA, pH 7.4 and lysed in TRIzol Reagent (Invitrogen) by mechanical shearing. RNA was extracted using standard procedures (Sambrook, 1989). DNA was removed using the Turbo DNA-free (Ambion) kit. cDNA synthesis was performed using oligo(dT)₁₂₋₁₈ (Invitrogen) and moloney murine leukemia virus (M-MLV) reverse transcriptase in the presence of RNaseOUT RNase inhibitor (Invitrogen). The presence of A domains was confirmed using 2-RT-PCR/U (5'-CACATCGGATCATTTG-3') together with 1-SeqDel/D (5'-GCAATAGATAATATTGACGAAAAGG-3'), the presence of TS domains using 2-RT-PCR/U together with 6-CTS2/D (5'-CGCGGATCCGATTTTGGTGAATGGAGC-3'). As a control, primers amplifying the unrelated gene *pbs28* were used.

2.5 Western blotting

Polyclonal antibodies against CTRP ($\alpha\text{-CT}^{\text{CTRP}}$) were raised in a half-lop rabbit using the recombinant GST-tagged cytoplasmic tail of CTRP (plasmid for producing recombinant antigen was kindly provided by Prof. M. Yuda, Japan). For ookinete cultures, white blood cells were removed from the infected blood using Plasmodipur filters (Euro-Diagnostica). Cultured ookinetes were enriched by red blood cell lysis in 150 mM NH_4Cl , 10 mM KHCO_3 , 1 mM EDTA, pH 7.4 and purified on 17.3% w/v Nycodenz (Nycomed) cushions in PBS. Ookinetes were lysed by boiling for 10 min in reducing SDS-buffer. Proteins from $4 \cdot 10^5$ or 10^5 ookinetes for detection with $\alpha\text{-CT}^{\text{CTRP}}$ or monoclonal $\alpha\text{-Pbs28}$ antibody (13.1, Winger et al., 1988) respectively were fractionated in 4-15% Tris-HCl gradient gels

(Biorad). SDS-PAGE and Western blotting was performed using standard methods (Sambrook, 1989). Detection was performed using the enhanced chemoluminescence Plus Western Blotting Detection System (GE Healthcare).

2.6 Immunofluorescence assay (IFA) of ookinetes

Thin blood films of cultured ookinetes were fixed in acetone/dry ice. The slides were then blocked in 1% bovine serum albumin (BSA)/PBS for 20 min and dried. Fixed ookinetes were labelled with antigen affinity-purified α -CT^{CTRP} antibodies at 6 μ g/ml in 1% BSA/0.1% Triton X-100/PBS and detected with α -rabbit-Alexa488 (Molecular Probes) in 1% BSA/0.1% Triton X-100/PBS. Imaging was performed using a Leica DMR microscope and a Zeiss AxioCam HRC camera using the Zeiss AxioVision software. Raw images were further processed with ImageJ version 1.43u.

2.7 Mosquito transmission assay

A. stephensi SD 500 and *A. gambiae* G3 were reared and maintained on fructose or sucrose respectively as previously described (Dimopoulos et al., 1998). For direct feeds, mice were infected with *P. berghei* bloodstages using standard protocols (Sinden, 2002). Three to four days post infection, direct mouse feeds of mosquitoes were performed as previously described (Sinden, 1997). For ookinete feeds, membrane feeders containing 800 ookinetes/ μ l suspended in blood of naïve mice were used. In all experiments, unfed mosquitoes were removed one day post infection. Oocysts were counted 10-12 days post infection by either phase-contrast or fluorescence microscopy. Melanised ookinetes were counted 5 days post infection by phase-contrast microscopy. Transmission intensities are expressed as the arithmetic mean of oocyst/ookinete numbers $\pm$ standard error of the mean (SEM).

2.8 Motility assay

Motility assays were performed as described (Moon et al., 2009). Ookinete movement was observed using a Leica DMR microscope and the Zeiss AxioCam HRC camera and processed with the Zeiss AxioVision software. Motility was expressed as the number of ookinete body lengths travelled during a 10 min period. At least 30 ookinetes were examined per preparation.

2.9 Immunofluorescence assay of infected midguts

Mosquitoes, fed on ookinetes, were dissected 4, 24 and 48 h post infection into PBS pH 7.2. The guts were fixed for 45 s in 4% formaldehyde/PBS pH 7.2 and rinsed in PBS before the blood meal was removed. Guts were further fixed for 30-45 min in 4% formaldehyde/PBS, washed in PBS and blocked in 1% BSA in PBS/0.1% Triton X-100. Parasites were labelled with the α -Pbs28 antibody 13.1 (Winger et al., 1988) and detected with Alexa488-conjugated α -mouse antibody (Molecular Probes). Imaging was performed on the Leica SP2 confocal microscope using the Leica Confocal Software version 2.61. Images were analysed using WCIF ImageJ version 1.37j and Volocity version 4.2.1 (Improvision). The basal side of the midgut epithelium was identified by locating the mosquito tracheae.

2.10 Gene silencing in *A. gambiae*

A 348 bp fragment of *A. gambiae* *ctl4* was amplified by PCR from cloned cDNA using the T7-promoter-containing primers CTL4 F T7 (5'-TAATACGACTCACTATAGGGTGGTTTGTATGCCGTGTCCT-3') and CTL4 R T7 (5'-TAATACGACTCACTATAGGGAATAAATTGTCTCGGTTTCATCATC-3'). dsRNA was generated using the MEGAscript T7 kit (Ambion) and purified with the RNeasy Mini kit (QIAGEN) using the manufacturers' protocols. The RNA was concentrated to 3 mg/ml and injected into mosquitoes 2-3 days prior to infection with *P. berghei* as previously described (Blandin et al., 2002).

3. Results

3.1 Generation and molecular analysis of transgenic parasites

To dissect the functional properties of the adhesive domains of CTRP, mutant *ctrp* lacking either all the A (A₆) or all the TS (TS₇) domains were cloned into a plasmid containing the human dihydrofolate reductase (*dhfr*) gene conferring resistance to the antimalarial drugs pyrimethamine and WR99210. Linear plasmid fragments were then transfected into either WT *P. berghei* ANKA clone 234 (kind gift from Prof. D. Walliker, University of Edinburgh) or into the *P. berghei* GFP-expressing line 507cl1, which constitutively expresses *gfp*, but does not contain a selectable marker (Janse et al., 2006a), for integration into the endogenous *ctrp* locus by double homologous recombination (Fig. 2A). Transgenic parasites were selected by subjecting the parasites to drug pressure and clonal lines were generated by limiting dilution cloning. Correct integration of the mutant *ctrp* alleles into the target locus was first verified by diagnostic PCR. PCR primers annealing to the 3'UTR of *ctrp* and to the *dhfr* gene respectively were used to detect the integrated plasmid by amplifying a 3.5 kb band (PCR1, Fig. 2B). When using primers annealing to the 3' UTR and the C-terminal part of CTRP, the expected size shift was observed between the originating and transgenic lines (PCR2, 5 kb versus 1.1 kb, Fig. 2B). Integration was further confirmed by Southern blot analysis. Parasite genomic DNA was digested with *Bst*BI and probed against the 3' UTR. The expected bands of 2.1 kb for the originating lines and 5.4 kb for the transgenic lines were identified (Fig. 2C). Transcription of mutant *ctrp* was verified by RT-PCR. The presence of the correct mRNA sequence was confirmed by detection of a 1.7 kb band (amplicon also contains the TS domains) or a 1.4 kb band probing for the A (RT-PCRA) or the TS (RT-PCRTS) domains respectively in WT ookinetes. Probing for the A domains in the TS₇ clones yielded 0.4 kb bands and probing for the TS domains in A₆ clone 4.10 a band of 1.4 kb as expected (Fig. 2D). Additionally, probing for TS domains in TS₇ lines, and for A domains in the A₆ confirmed the absence of the TS and A domains (data not shown).

Polyclonal antibodies against the cytoplasmic tail of CTRP (α -CT^{CTRP}, Yuda et al., 1999b) were used to identify WT and mutant CTRP by Western blot analysis. Full-length CTRP (215 kDa) was detected in the WT control strains 234 and 507cl1 (Fig. 2E). A protein of approximately 164 kDa was detected in TS₇ clones 9 and 10 (Fig. 2E) and in GFP/ TS₇ clone 15 (data not shown). An approximately 66 kDa protein could be detected in A₆ clone 4.10 (Fig. 2E) and a similar band was observed in GFP/ A₆ clone 5 (data not shown). The

sizes of the mutant CTRP proteins are consistent with the introduced deletions of the A and TS domains. Analyses of the band intensities showed that the transgenic parasites produced at least as much CTRP as their WT counterparts.

3.2 Localisation of mutant CTRP

To analyse the localisation of mutant CTRP on the transgenic parasite, IFA on ookinetes using affinity-purified α -CT^{CTRP} antibodies was performed. Ookinetes of WT and A₆ clone 4.10 displayed a similar and specific labelling pattern, indicating that both WT and A domain-deficient CTRP are similarly localised (Fig. 3). TS₇ clone 10 shows a similar albeit fainter labelling pattern. The negative control parasite CTRPko, which does not contain the cytoplasmic tail of CTRP (Dessens et al., 1999) and thus cannot be recognised by α -CT^{CTRP}, only displayed a faint background signal (Fig. 3). Secondary antibody alone did not recognise any of the parasite lines tested (data not shown) and all lines showed normal labelling with α -Pbs28 antibody (Fig. 3).

3.3 Mosquito infectivity of transgenic parasites

To study infectivity of the transgenic parasites to mosquitoes, oocyst numbers were counted 10-12 days post infection, and the infection levels were compared (Table 1). Infection levels of TS₇ clone 9 and clone 10 were found to be either lower or similar to WT infections (Experiments 1 and 3). A₆ clone 4.10 was completely non-infectious to mosquitoes (Experiments 2 and 3). To rule out artefacts of transfection and selection, mosquitoes were also fed on mice infected with the independently obtained GFP-expressing mutant lines (Table 1, Experiment 4). GFP/ TS₇ clone 15 showed comparable infectivity to the GFP/WT line; in contrast, GFP/ A₆ clone 5 yielded no oocysts.

No aberration in ookinete formation in the transgenic parasites studied here was detected, which is consistent with CTRPko parasites producing normal numbers of ookinetes (Dessens et al., 1999; Yuda et al., 1999a). To rule out that the reductions in oocyst numbers observed above were not the result of variable gametocyte numbers, we fed cultured ookinetes at equal densities to mosquitoes (Experiment 5). Infections with TS₇ clones yielded no significant differences compared to WT infections, whereas A₆ clones were again unable to produce oocysts. To test whether the observed phenotypes were specific to *A. stephensi*, infections with *A. gambiae* were performed using the GFP-expressing mutant lines (Experiments 6 and 7). GFP/ TS₇ clone 15 showed again similar infectivity to WT, whereas GFP/ A₆ clone 5 did not produce any oocysts. Thus, the results obtained with *A. stephensi* infections were further confirmed and specificity of the phenotypes to *A. stephensi* was ruled out. These combined observations indicate that the A domains of CTRP are essential for *P. berghei* transmission in the mosquito, whilst its TS domains are functionally redundant in both *A. stephensi* and *A. gambiae* mosquitoes.

3.4 Motility of transgenic parasites

The motility of TS₇ clone 9 and A₆ clone 4.10 ookinetes was observed by light microscopy in an in-gel matrigel-based assay (Moon et al., 2009) and was compared to the motility of WT and CTRPko ookinetes. CTRPko ookinetes displayed non-productive motility such as bending and flexing, but no locomotive gliding motility (Fig. 4A,B and

Supplementary video 4) as previously reported (Dessens et al., 1999). Movement of TS₇ ookinetes was indistinguishable from WT ookinetes, displaying typical cork-screw locomotion (Fig. 4A and Supplementary videos 2 and 1). Similar distances were covered by WT and TS₇ ookinetes (median of 4.8 [mean 4.6] body lengths/10 min for WT and 4.6 [mean 4.3] body lengths/10 min for TS₇ clone 9, p-value 0.606, Fig. 4B). In contrast, A₆ clone 4.10 ookinetes showed severely limited locomotion (Fig. 4A and Supplementary videos 3): less than 50% of the ookinetes in A₆ mutants displayed any gliding motility and the velocity of those that moved was significantly reduced compared to WT (median of 0.0 [mean 0.6] body lengths/10 min, p-value < 0.0001, Fig. 4B).

3.5 Invasion of the midgut epithelium

To investigate the cause of the lack of oocyst development in the A₆ lines, ookinete feeds were performed and midgut infections were observed by confocal microscopy at 4 hrs post infection by antibody detection of the ookinete surface protein Pbs28. WT and TS₇ clone 9 parasites were clearly observed in the midgut epithelium (Fig. 5). Their presence in the epithelial layer was confirmed by z-stack projections of confocal micrographs. In contrast, A₆ clone 4.10 and CTRPko parasites were never observed inside the midgut epithelium (Fig. 5). We further analysed gut infections 24 and 48 hours post infection by light microscopy. Mosquitoes with WT and TS₇ infections revealed that most, or all of the guts were infected with young oocysts; conversely, mosquitoes infected with A₆ or CTRPko only sporadically revealed parasites at 24 hours post infection, and no parasites at 48 hours post infection (Table 2 and data not shown). Ookinetes have previously been detected in guts infected with CTRPko ookinetes, but these were shown by confocal microscopy to be only transiently associated with the midgut (data not shown). These combined observations indicate that A domain-deficient ookinetes do not invade the mosquito midgut epithelium, similar to CTRPko ookinetes (Dessens et al., 1999).

To confirm that A₆ ookinetes are unable to invade mosquito midguts, infections were also performed in *A. gambiae* G3 mosquitoes in which the inhibitor of melanisation C-type lectin 4 (CTL4) was silenced. In the absence of CTL4, all ookinetes that invade the midgut epithelium are melanised (Osta et al., 2004). Midguts infected with WT and TS₇ clone 9 parasites showed a high number of melanised ookinetes (Fig. 6 and Table 3); infection of midguts with TS₇ clone 9 yielded similar or lower levels to infections with WT parasites. In sharp contrast, CTRPko and A₆ clone 4.10 parasite-infected midguts only sporadically contained few melanised ookinetes (Fig. 6, Table 3). The comparable prevalence and intensity of infection in CTRPko and A₆ parasites confirm that A₆ ookinetes are severely impaired in their ability to invade the mosquito midgut epithelium.

4. Discussion

Here, we report the first structure/function analysis of the adhesive domains of the essential ookinete protein CTRP. Using genetically modified parasites lacking either all the A domains or, separately, all the TS domains of CTRP we demonstrate that the A domains of CTRP are essential for the infectivity of *P. berghei* ookinetes, while the CTRP TS domains are redundant. Whilst removing all the A domains of CTRP results in a complete block of

transmission, where no oocysts are observed on *Anopheles* midguts, removing all the TS domains yields infections lower or similar to WT infections (Table 1, 3). However, if ookinete numbers are controlled, the TS₇ parasites infect at equal levels to WT (Table 1, Experiment 5) confirming that the TS domains are redundant. We believe that variability in infection by direct feeding is due to the differences in infection of the mammalian host and that TS₇ parasites behave similar to WT; this hypothesis is confirmed by the observation that motility, attachment to midguts and the ability to invade midguts are comparable between WT and TS₇ (Fig. 4, 5 and 6). The observed redundancy of the CTRP TS domains is unexpected and contrasts the previously identified role of the TS domain of TRAP in sporozoite motility (Wengelnik et al., 1999), invasion of mosquito salivary glands (Wengelnik et al., 1999; Matuschewski et al., 2002) and infectivity to the vertebrate host (Matuschewski et al., 2002), raising questions as to why the CTRP molecule has retained these repeated, yet non-essential domains. The existence of various pathways of liver cell adherence of sporozoites revealed by the use of TRAP mutants suggests that the TRAP A domain mediates adhesion in a GAG-independent pathway, whereas the TS domain uses a GAG-dependent pathway (Matuschewski et al., 2002). TREP on the other hand, a protein important for sporozoite motility in the mosquito and salivary gland invasion, contains only a single TS domain in its large extracellular region (Kaiser et al., 2004). Several members of the TRAP family contain more than one TS domain (Fig. 1, reviewed in Morahan et al., 2009), i.e. *Eimeria tenella* 100 kDa protein (Etp100, Tomley et al., 1991), *Cryptosporidium* TRAP-C1 (Spano et al., 1998), *T. gondii* micronemal protein 2 (MIC2, Wan et al., 1997) and *N. caninum* MIC2 (Lovett et al., 2000), which all contain six TS domains. It is noteworthy that the canonical VTCG motif of the TS domains conserved between human thrombospondin type 1, CSP and TRAP is modified to XCTG in CTRP where amino acid X is not conserved (Trottein et al., 1995). The VTCG motif in other adhesive proteins is a strong attachment factor, and adhesion can be inhibited by competing sulphated glycoconjugates (Prater et al., 1991). The alteration of the VTCG motif may result in variation of binding affinity and differences in ligand specificity. Another conserved motif, the cluster of basic residues C-terminal to the VCTG motif (present in all the seven TS domains of CTRP) was shown in CSP to be important to the binding of heparan sulphate proteoglycans present on hepatocytes (Sinnis et al., 1994). It remains to be established whether the functional redundancy of the CTRP TS domains is common to all *Plasmodium* species. Functionality/redundancy may also be dependent on insect vector species other than *A. stephensi* and *A. gambiae*, as they may present different ligands or substrates for CTRP to interact with.

Uniquely, CTRP is the only member of the TRAP family containing more than one conserved A domain (TLP contains one conserved and one very divergent A domain, Moreira et al., 2008). Why has CTRP evolved to contain 6 copies of A domains? Arguably, binding can be enhanced by increasing avidity mediated by multiple binding sites/domains. Therefore, we hypothesise that the mosquito ligands presented to CTRP in the gut, and possibly later at the basal lamina, may be of low affinity and therefore necessitate replication, in contrast to TRAP binding to salivary gland ligands. Parasites carrying mutant CTRP with different numbers of A domains could be utilised to confirm this hypothesis.

CTRP, as yet the only member of the TRAP family identified in the ookinete, represents the critical link between the substratum and the molecular motor of the ookinete having been shown to bind aldolase (Heiss et al., 2008) that in turn, interacts with actin of the motor complex. Cytochalasin D, an inhibitor of the actin/myosin motor, blocks the translocation of CTRP to the posterior pole of the ookinete (K. Lal and M. J. Delves, unpublished). The role of CTRP in motility was previously demonstrated by knock-out studies (Dessens et al., 1999). Deletion of all the CTRP A domains results in very limited locomotion of the ookinetes, demonstrating the importance of these domains in ookinete motility. This observation contrasts with studies of TRAP showing the A domain not to be necessary for sporozoite gliding motility (Wengelnik et al., 1999; Matuschewski et al., 2002). Observations on sporozoites carrying a mutation in the TRAP TS domain produced contradictory data as to whether this domain is involved in sporozoite gliding motility (Wengelnik et al., 1999; Matuschewski et al., 2002). On their own, the TS domains of CTRP render ookinetes ineffectually motile, indicating that these domains make only a very small contribution to ookinete progressive locomotion. The inability of A₆ ookinetes to develop into oocysts could simply be due to their inability to reach or cross the midgut epithelium as a result of impaired motility. A₆ ookinetes may have lost their adhesive properties required to gain sufficient traction upon the substratum to facilitate locomotion, and/or to bind effectively to the midgut epithelium prior to invasion.

Surprisingly, our studies of midgut infection in refractory mosquitoes show that CTRPko and A₆ parasites occasionally give rise to small numbers of melanised ookinetes. This finding was unexpected since in order to be melanised, ookinetes need to reach the basal side of the midgut epithelium. Melanised ookinetes were not observed in a previous study of CTRPko infection in refractory L3-5 *A. gambiae* mosquitoes (Dessens et al., 1999). It is possible that small numbers may reach the midgut epithelium passively, but it is unknown how, in the absence of active gliding motility, an ookinete would be able to reach the basal side of the midgut. Nevertheless, the results presented here confirm that A₆ ookinetes are severely impaired in their ability to invade the mosquito midgut epithelium.

TRAP family proteins are members of a structurally conserved family of proteins which are involved in invasion, adhesion and motility (Supplementary table). To date, three *Plasmodium* TRAP family members are known for the sporozoite, one for the merozoite and one for the ookinete. The sporozoite encounters two hosts and invades different cell types which may necessitate the presence of different TRAP family proteins. As the transition from ookinetes to oocysts is a major bottleneck in the *Plasmodium* life cycle, this process could potentially be targeted to reduce parasite transmission. Accordingly, CTRP as an essential protein for the ookinete-to-oocyst transition constitutes a potential target for transmission-blocking drugs or antibodies. Our results presented here show that the A domains of CTRP provide the most promising part of the molecule to develop potential transmission intervention strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic representation of the domain structure of the TRAP family.

The TRAP-family comprises of proteins containing a signal peptide, at least one TS domain and a transmembrane domain. The size of the proteins is given on the right and the diagram is in scale except for TREP. PBANKA_070790 is a PlasmoDB identification number. CSP was previously regarded as a member of the TRAP family (Naitza et al., 1998), but due to the membrane attachment being mediated by a C-terminal GPI anchor it has been excluded. Proteins from various species/genera are colour coded as follows: black: *Plasmodium*, brown: *Babesia bovis*, green: *Eimeria*, red: *Toxoplasma gondii*, orange: *Neospora caninum*, blue: *Cryptosporidium*. TRAP: thrombospondin-related adhesive protein, BTRAP: *Babesia bovis* TRAP, Etp100: *Eimeria tenella* 100 kDa protein, TgMIC2: *Toxoplasma gondii* micronemal protein 2, NcMIC2: *Neospora caninum* MIC2, TLP: TRAP-like protein, CTRP: circumsporozoite protein- and TRAP-related adhesive protein, MTRAP: merozoite TRAP, PTRAMP: *Plasmodium* thrombospondin-related apical merozoite protein, TRAP-C1: TRAP-C1 from *Cryptosporidium*, TRSP: thrombospondin-related sporozoite protein, TREP: TRAP-related protein, CpTSP7: *Cryptosporidium parvum* thrombospondin-related protein 7 (there are 10 other *C. parvum* genes containing TS domains (Deng et al., 2002), however, only CpTSP7 is listed as the others are not compliant with the criteria used here. Note that *P. falciparum* orthologue of PBANKA_070790 contains 2 TM domains; in the *P. berghei* protein, the signal peptide and the first TM domain overlap.

Fig. 2. Generation of transgenic parasites and molecular analysis of mutant CTRP.

(A) Schematic diagram of the double homologous recombination event replacing endogenous *ctrp* with mutant *ctrp*. The crosses show the sites of crossing-over; dashed lines show the part of the plasmid inserted into the *ctrp* locus. Arrows represent primers used for diagnostic PCRs; forward arrows used for the two different PCR reactions (Fig. 2B) are labelled. The yellow blocks show the probe used for detection after Southern blotting. *Bst*BI restriction sites used for the Southern blot are marked. Arrowheads indicate the annealing sites for RT-PCR primers. Colour codes indicate following parts of the gene: blue: 5'/3' UTR of *ctrp*, black: signal sequence, red: all A domains, green: all TS domains, white: transmembrane and cytoplasmic domains, brown: 5'/3' UTR of *pbdhfr*, orange: *hdhfr*. (B) Diagnostic PCR analysis of the resulting clonal lines ('clone' is abbreviated to 'cl'). The top panel shows the diagnostic PCR probing for the *hdhfr* after integration (Fig. 2A), thus confirming the plasmid's insertion into the genome. The bottom panel shows the diagnostic PCR encompassing the cytoplasmic end of *ctrp* and the 3' UTR (Fig. 2A). After integration of the plasmid, the amplification of a DNA fragment containing the inserted *hdhfr* cassette results in a size shift from 1.1 kb to 5 kb. Expected sizes of bands are marked on the right. PCR with an arbitrary gene (guanyl cyclase β) was used as a positive control. (C) Southern blot analysis of transgenic parasites. The probe used is indicated in Fig. 2A. Integration of the plasmid fragment results in a shift from 2.1 kb to 5.4 kb. Expected band sizes are indicated on the right hand side of the panel. (D) RT-PCR analysis of ookinetes from transgenic strains. Primers used are shown in Fig. 2A. The top panel shows the result if probing for the A or the TS domains respectively. mRNA from the WT was probed for both domain types. As a negative control, reactions without reverse transcriptase were performed. The ookinete surface gene *pbs28* was used as a positive control (bottom panel). On the right hand side, expected band sizes are listed. (E) Western blot analysis of mutant CTRP proteins using the C-terminal polyclonal antibody against CTRP (Yuda et al., 1999a). *Pbs28* detected

by the monoclonal antibody 13.1 was used as a loading control. Expected band sizes are indicated on the right hand side of the panel.

Fig. 3. Immunofluorescence of ookinetes.

Cultured, acetone-fixed ookinetes were labelled with polyclonal α -CTCTRP antibody, which recognises the cytoplasmic tail of CTRP. Subsequently, the antibodies were detected using secondary antibodies conjugated to Alexa488. Unspecific rabbit IgG was used as a negative control and α -Pbs28 as a positive control. Shown are phase contrast images on the left and a merged image between the green and the blue (DAPI) channel. 'Clone' is abbreviated to 'cl'. Scalebar = 5 μ m.

Fig. 4. Motility of TS_7 and A_6 ookinetes.

(A) Series of images (taken 2 min apart) showing a representative path of movement that transgenic ookinetes covered in 8 minutes compared to paths covered by WT and CTRPko ookinetes. The total path is marked with a dashed pink line. Scalebar = 10 μ m. (B)

Distribution of ookinete path lengths (in multiple body lengths) covered in 10 min. Median path lengths are represented as horizontal bars. n is the number of ookinetes observed.

Fig. 5. IFA on midguts 4 h after infection with ookinetes.

Ookinets were stained with α -Pbs28 antibody. Shown are overlays of confocal image stacks after differential interference contrast (DIC) and fluorescence acquisitions. The yz- and xz-projections are shown on the left or top respectively. The cutline for the projections are shown on the image stack. Scalebar = 20 μm .

Fig. 6. Ookinete invasion of midguts.

A. gambiae G3 mosquitoes were treated with dsRNA against CTL4 prior to infection with *P. berghei* carrying mutant CTRP. Melanised ookinetes on midguts were observed at 5 days post infection. Insets show magnifications of melanised ookinetes. Scalebars = 100 μ m/20 μ m (inset).

Table 1
Transmission of transgenic parasites by *A. stephensi* (As) or *A. gambiae* (Ag).

Experiment	Mosquito	Feed	Parasite	n	Prevalence	Mean oocysts $\pm$ SEM	p-value ^a
1	As	Mouse 1	WT	40	80%	71.3 $\pm$ 19.6	-
	As	Mouse 2	TS ₇ clone 9	48	88%	60.0 $\pm$ 14.4	0.946
	As	Mouse 3	TS ₇ clone 10	34	65%	26.4 $\pm$ 7.8	0.028
2	As	Mouse 1	WT	10	80%	108.6 $\pm$ 41.1	-
	As	Mouse 2	A ₆ clone 4.10	18	0%	0 $\pm$ 0	< 0.001
3	As	Mouse 1	WT	31	87%	102.3 $\pm$ 21.7	-
	As	Mouse 2	TS ₇ clone 9	50	58%	44.5 $\pm$ 11.3	0.007
	As	Mouse 3	TS ₇ clone 10	36	72%	31.9 $\pm$ 8.6	0.013
	As	Mouse 4	A ₆ clone 4.10	50	0%	0 $\pm$ 0	< 0.001
4	As	Mouse 1	GFP	20	100%	126.4 $\pm$ 18.4	-
	As	Mouse 3	GFP/ TS ₇ clone 15	30	100%	119.3 $\pm$ 12.1	0.965
	As	Mouse 5	GFP/ A ₆ clone 5	34	0%	0 $\pm$ 0	< 0.001
5	As	Ookinete	WT	43	100%	34.6 $\pm$ 3.2	-
	As	Ookinete	TS ₇ clone 9	25	100%	49.6 $\pm$ 9.4	0.726
	As	Ookinete	TS ₇ clone 10	34	97%	41.1 $\pm$ 5.2	0.587
	As	Ookinete	A ₆ clone 4.10	38	0%	0 $\pm$ 0	< 0.001
6	Ag	Mouse 1	GFP	41	59%	31.6 $\pm$ 10.7	-
	Ag	Mouse 2	GFP/ TS ₇ clone 15	29	43%	22.0 $\pm$ 8.4	0.406
	Ag	Mouse 3	GFP/ A ₆ clone 5	50	0%	0 $\pm$ 0	< 0.001
7	Ag	Mouse 1	GFP	34	38%	62.2 $\pm$ 22.6	-
	Ag	Mouse 2	GFP/ TS ₇ clone 15	18	39%	45.6 $\pm$ 15.3	0.983
	Ag	Mouse 3	GFP/ A ₆ clone 5	59	0%	0 $\pm$ 0	< 0.001

^a determined by comparing the oocyst number medians by Mann Whitney U test

Table 2
Infectivity of ookinetes to *A. stephensi* 24 hrs and 48 hrs after membrane feeding.

Listed are the number of midguts found to contain attached ookinetes (positive) or not (negative).

Parasite	24 hours post infection			48 hours post infection		
	positive	negative	total midguts analysed	positive	negative	total midguts analysed
WT	15	2	17	6	0	6
TS ₇ clone 9	10	2	12	17	2	19
TS ₇ clone 10	17	6	23	16	6	22
A ₆ clone 4.10	2	19	21	0	8	8
CTRPko	0	18	18	0	7	7

Table 3
Penetration of *A. gambiae* (Ag) midguts by transgenic ookinetes after knock-down of *A. gambiae* CTL4.

Experiment	Mosquito	Feed	Parasite	n	Prevalence	Mean ookinetes $\pm$ SEM	p-value ^a
1	Ag	Mouse 1	WT	36	97%	288.0 $\pm$ 44.2	-
	Ag	Mouse 2	TS ₇ clone 9	48	98%	179.9 $\pm$ 30.6	0.0329 ^b
	Ag	Mouse 3	A ₆ clone 4.10	47	30%	1.43 $\pm$ 0.54	0.0644 ^c
	Ag	Mouse 4	CTRPko	41	20%	0.41 $\pm$ 0.13	-
2	Ag	Mouse 1	WT	40	93%	140.3 $\pm$ 24.5	-
	Ag	Mouse 2	TS ₇ clone 9	26	77%	167.3 $\pm$ 35.5	0.8903 ^b
	Ag	Mouse 3	A ₆ clone 4.10	51	8%	0.08 $\pm$ 0.04	0.1955 ^c
	Ag	Mouse 4	CTRPko	59	12%	0.25 $\pm$ 0.09	-

^a determined by comparing the medians of the ookinete numbers by Mann Whitney U test;

^b comparison to WT;

^c comparison to CTRPko.