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CTRP is essential for mosquito infection by malaria ookinetes

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The malaria parasite suffers severe population losses as it passes through its mosquito vector. Contributing factors are the essential but highly constrained developmental transitions that the parasite undergoes in the mosquito midgut, combined with the invasion of the midgut epithelium by the malaria ookinete (recently described as a principal elicitor of the innate immune response in the *Plasmodium*-infected insect). Little is known about the molecular organization of these midgut-stage parasites and their critical interactions with the blood meal and the mosquito vector. Elucidation of these molecules and interactions will open up new avenues for chemotherapeutic and immunological attack of parasite development. Here, using the rodent malaria parasite *Plasmodium berghei*, we identify and characterize the first microneme protein of the ookinete: circumsporozoite- and TRAP-related protein (CTRP). We show that transgenic parasites in which the *CTRP* gene is disrupted form ookinetes that have reduced motility, fail to invade the midgut epithelium, do not trigger the mosquito immune response, and do not develop further into oocysts. Thus, CTRP is the first molecule shown to be essential for ookinete infectivity and, consequently, mosquito transmission of malaria.

Keywords: microneme/mosquito/*Plasmodium berghei*/transgenic parasite

Introduction

Within 24 h of entering the mosquito the malaria parasite undergoes rapid production of gametes, fertilization and transition of the zygote into the motile ookinete. The ookinete escapes the hostile environment in the midgut lumen by invading and traversing the midgut epithelium. Under the basal lamina on the outer, haemolymph side of the midgut, the ookinete develops further into the oocyst (Sinden *et al*., 1985). Like the other invasive stages of *Plasmodium* (sporozoite and merozoite) and other apicomplexan parasites, the ookinete contains specialized secretory organelles (rhoptries and micronemes) at its apical end (known as the apical complex), components of which are considered to play a role in host cell recognition and attachment as well as parasite gliding motility (Dubremetz *et al*., 1993). Although the ookinete is a demonstrated target of transmission-blocking immunity (Sinden, 1994; Kaslow, 1997), our knowledge of the ookinete at the molecular level is limited to two surface antigens of the P25/28 family (Duffy and Kaslow, 1997) and a secreted chitinase (Shahabuddin *et al*., 1993). Very recently, the expression in the ookinete of a new molecule of *Plasmodium berghei* named circumsporozoite- and TRAP-related protein (CTRP) was reported (Yuda *et al*., 1999). Similar findings in our laboratory prompted us to undertake a functional analysis of this molecule.

CTRP, which has a structural homologue in the human malaria parasite *Plasmodium falciparum* (Trottein *et al*., 1995), has a predicted structure composed of six von Willebrand factor type A-related and seven human thrombospondin type I-related adhesive domains, and belongs to the family of thrombospondin-related proteins of apicomplexan parasites. This family includes proteins of the medically and veterinary important pathogens *Toxoplasma* (MIC2), *Cryptosporidium* (TRAP-C1) and *Eimeria* (Etp100) (Naitza *et al*., 1998). In addition to the adhesive domains, these proteins contain, with the exception of *Plasmodium* circumsporozoite protein, a conserved C-terminal transmembrane domain and a short cytoplasmic tail. For those members characterized, their structural features, along with their organellar localization patterns (in the apical complex), suggest that they may play an important role in the process of parasite invasion of target cells (Naitza *et al*., 1998). To investigate the role of CTRP we constructed transgenic parasites in which the *CTRP* gene is disrupted. Transmission experiments with these parasites show that CTRP is essential for ookinete invasion of the midgut epithelium and oocyst formation. Its role in ookinete to oocyst development will be discussed.

Results

Identification of the CTRP gene

We generated a subtracted cDNA library corresponding to mRNA from combined midgut-stage *P.berghei* parasites (gametes, zygotes and ookinetes). An abundant clone (clone 2) was identified as a partial, 1080 bp cDNA corresponding to the structural homologue of *P.falciparum* CTRP (Trottein *et al*., 1995) by protein database homology search (BLASTP) and was later shown to correspond to the *P.berghei CTRP* gene (Yuda *et al*., 1999). This sequence was used for gene targeting and as a molecular probe.

Expression and subcellular localization of CTRP

Transcription of the *CTRP* gene was assessed by Northern blot analysis. A mRNA of ~7 kb was detected in combined

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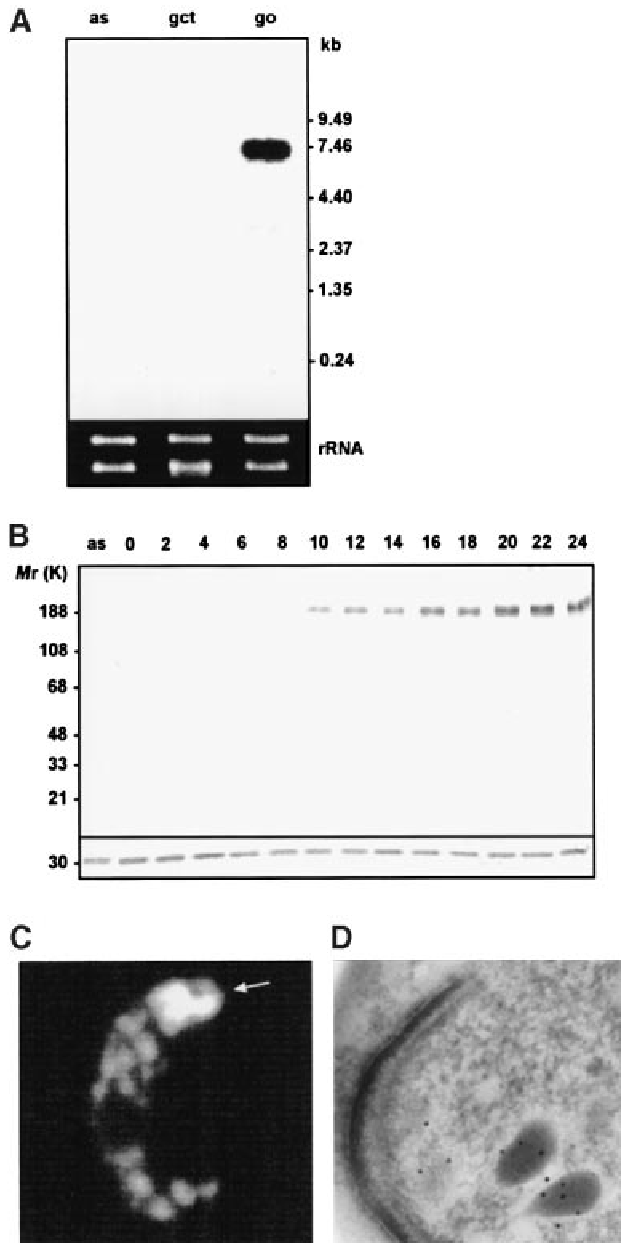

Fig. 1. CTRP expression and localization in *P.berghei* parasites using mAb 3A5. (**A**) Northern blot analysis of total RNA purified from asexual blood-stage parasites (as), gametocytes (gct) and combined midgut stages (go). RNA amounts have been normalized using the large and small subunit rRNAs (lower panel). (**B**) Western blot analysis of parasites purified from 24 h *in vitro* ookinete cultures at 2 h time intervals (0–24) and from infected blood (as). Protein amounts have been normalized using a mAb to a M_r ~30 000 housekeeping protein (lower panel). (**C**) Immunofluorescent antibody staining of a typical *in vitro* cultured WT ookinete. The arrow points to the apical end of the cell. (**D**) Immunogold electron microscopy of an ookinete thin section showing the anterior end with labelled micronemes.

midgut stages (Figure 1A). After prolonged exposure a signal was also detected in gametocytes (data not shown), while in asexual blood-stage parasites no signal was detected (Figure 1A). These observations indicated that transcription of the *CTRP* gene is restricted to midgut-stage parasites. The low levels of CTRP messenger in the gametocyte sample we suspect are the result of partial gametocyte activation during purification.

Expression of CTRP was assessed by Western blot analysis of parasites purified from *in vitro* ookinete cultures at 2 h time intervals post-exflagellation, using the CTRP-specific monoclonal antibody (mAb) 3A5. A protein of M_r ~215 000 was detected from 10 h onwards in this time course and its expression level increased steadily until the 24 h time point studied (Figure 1B). This expression pattern is consistent with ookinete development in *in vitro* cultures and indicated that CTRP is expressed in the ookinete stages of *P.berghei*.

Indeed, immunolocalization of CTRP in formaldehyde-fixed parasites gave intracellular staining only in ookinetes, which was concentrated at the apical end in most ookinetes examined (Figure 1C). This observation indicated that CTRP may be targeted to the apical organelles, as has been demonstrated for other members of the thrombospondin-related apicomplexan protein family (Naitza *et al*., 1998). This was confirmed by immunogold electron microscopy of ookinetes, which identified micronemes as target organelles (Figure 1D). CTRP is thus the first microneme protein of the ookinete identified. This subcellular localization pattern pointed to a role of CTRP in ookinete invasion of the midgut epithelium.

Construction and molecular analysis of knockout parasites

To study the role of CTRP, gene knockout parasites were generated. This was achieved by inserting a modified *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase gene (*TgDHFR/TS*) into the *CTRP* locus at nucleotide position 4955 by double cross-over homologous recombination (Figure 2A). *TgDHFR/TS* confers resistance to the antimalarial drug pyrimethamine. The partial, 1080 bp *CTRP* sequence of clone 2 available to us was used to flank the *TgDHFR/TS* cassette and allow homologous recombination. Subsequently, two clones (numbered 31 and 45) derived from independent recombination events were studied to eliminate possible effects from clonal phenotypic variation.

Correct integration of the *TgDHFR/TS* cassette into the *CTRP* gene was verified by Southern blot analysis of *Sph*I-digested genomic DNA (Figure 2B). Hybridization with a *CTRP*-specific probe (no *Sph*I site present) gave rise to a single band in the parental (WT) parasite sample but two bands in the knockout (KO) parasite samples (Figure 2B). This demonstrated a single insertion in the *CTRP* gene of the *TgDHFR/TS* cassette that contains four *Sph*I sites (Figure 2A). The latter was confirmed by using a *TgDHFR/TS* probe, which detected three specific fragments in the KO samples and no signal in the WT sample (Figure 2B). These results confirmed correct integration of the selection marker in the target site, and showed that clones 31 and 45 are genetically indistinguishable.

Knockout parasites developed gametocytes and formed ookinetes *in vitro* in similar numbers to, and morphologically indistinguishable from, WT parasites in Giemsa-stained preparations (data not shown). However, when analyzed by Western blotting with mAb 3A5 a smaller protein of M_r ~190 000 was detected in KO parasites compared with that detected in WT parasites (Figure 2C). This protein must result from translation of truncated mRNA transcribed from the *CTRP* gene in the transgenic

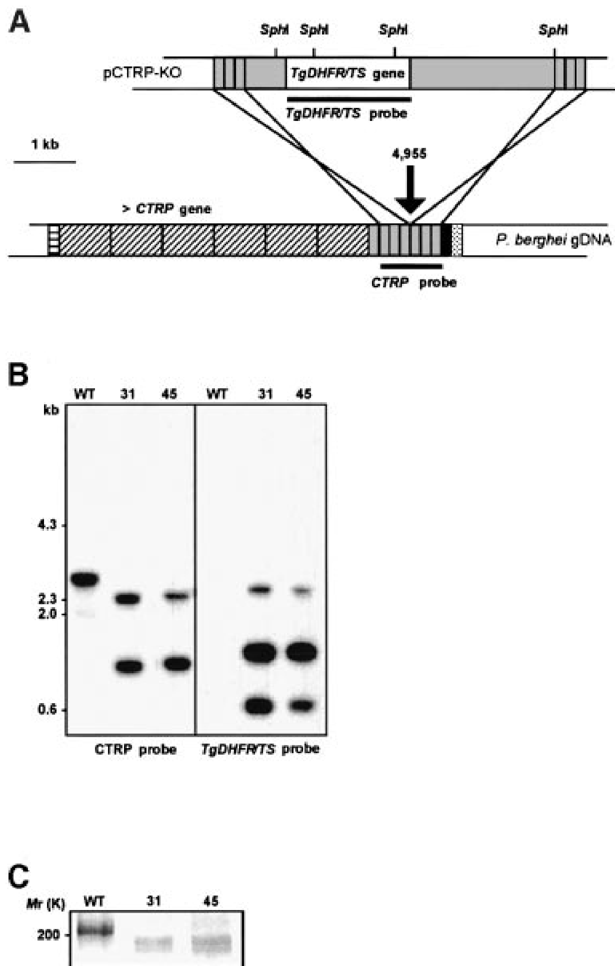

Fig. 2. Transgenic parasite construction and molecular analysis. (**A**) Schematic diagram of the *CTRP* locus on *P.berghei* genomic DNA (gDNA), the transfection vector pCTRP-KO containing the *TgDHFR/TS* cassette, and the double homologous recombination cross-over sites (crossed lines). The arrow shows the integration site at nucleotide position 4955. Indicated are the *TgDHFR/TS* gene (white), *P.berghei* flanking sequences (light grey), thrombospondin type I-related domains (dark grey), von Willebrand factor type A-related domains (diagonal stripes), transmembrane domain (black), cytoplasmic tail (dotted), signal peptide (horizontal stripes), *Sph*I restriction sites and the molecular probes used in Southern blotting. (**B**) Southern blot analysis of *Sph*I-digested gDNA from WT parasites and KO clones 31 and 45. (**C**) Western blot analysis of combined midgut stages of WT parasites and KO clones 31 and 45, using mAb 3A5.

parasites. It is expected to be 266 amino acids smaller as a result of the *TgDHFR/TS* insertion (Figure 2A). In addition, the protein bands were of lower intensity than those in the WT sample, suggesting that the truncated CTRP messenger or gene product is less stable than its WT equivalents.

Immunolocalization of CTRP in KO ookinetes revealed another difference with WT ookinetes. Whilst an intracellular staining similar to that in WT ookinetes was observed, the concentration of stain at the apical end of the ookinetes was absent and immunogold electron microscopy failed to label micronemes (data not shown). These observations suggest that the truncated form of CTRP is not targeted to the micronemes. This may be the result of incorrect folding of the truncated protein or the absence of targeting sequences in the C-terminal region.

Mosquito infectivity of knockout parasites

If CTRP played a role in invasion of the midgut epithelium we would anticipate a reduction in ookinete infectivity to the mosquito and, consequently, a reduction in the number of oocysts developing. The infectivity of the parasites to *Anopheles stephensi* mosquitoes was assessed by counting oocyst numbers on the midgut at 10 days following feeding on gametocyte-infected rodents, and following feeding on *in vitro* cultured ookinetes presented in membrane feeders. In sharp contrast to WT parasites, neither type of feed of the KO parasites resulted in oocyst development (Table I). Consequently, mosquitoes infected with KO parasites were non-infectious to mice. The absence of any difference between gametocyte and ookinete feeds (Table I) suggested that CTRP has no role in ookinete development *in vivo*. Indeed, we observed fully developed ookinetes in midguts at 24 h post-infection. From these findings we conclude that CTRP is essential for ookinete to oocyst transition of *P.berghei* in *A.stephensi*. The distinct phenotype of the KO parasites indicates that the truncated form of CTRP is unable to fulfil its biological role.

Midgut invasion by knockout ookinetes

We examined further mosquito midguts at 24 h post-infection for the presence of ookinetes. After removal of the blood meal from dissected, gametocyte-infected *Anopheles gambiae* midguts, WT ookinetes were found in the epithelium. In sharp contrast, no KO ookinetes were observed. Similarly, when we examined dissected, infected *A.stephensi* midguts for the presence of ookinetes or young oocysts on the basal surface, we observed WT but never KO parasites. We then infected *A.gambiae* of a malaria-refractory strain, which melanizes ookinetes and young oocysts in the midgut wall (Collins *et al*., 1986). In this experiment, whilst these mosquitoes showed numerous encapsulated WT parasites, none were found after infection with KO parasites (Figure 3A). Finally, we tested for induction of the mosquito's innate immune response upon infection. This normally occurs at 24 h post-infection when malaria ookinetes traverse the midgut epithelium (Dimopoulos *et al*., 1998). Within experiments, an upregulation of the immune markers defensin and Gram-negative binding protein (GNBP) was observed in mosquitoes infected with the WT parasites, while no upregulation was observed in KO-parasite-infected mosquitoes (Figure 3B). Taken together, these observations show that the KO ookinete seems unable to invade the midgut epithelium *in vivo*.

Locomotion of knockout ookinetes

CTRP is structurally related to *Plasmodium* TRAP (thrombospondin-related adhesive protein), another member of the thrombospondin-related apicomplexan protein family. *TRAP* is expressed in the sporozoite stages and encodes only one von Willebrand factor type A-related adhesive domain and only one thrombospondin type I-related adhesive domain (Robson *et al*., 1988). There is experimental evidence that TRAP plays a role in sporozoite gliding motility on glass surfaces (Spaccapelo *et al*., 1997; Sultan *et al*., 1997). Hence, a role of CTRP in gliding motility of the ookinete was conceivable. To assess this, initially we used an assay described for sporozoite gliding motility

Table I. Transmission of CTRP gene knockout parasites by *A.stephensi*

Experiment	Type of feed[a]	Parasite	No. of mosquitoes dissected	% Infection	Transmission (mean oocyst No. ± SEM)
1	mouse 1	KO 31	50	0	0 ± 0
	mouse 2	KO 31	50	0	0 ± 0
	mouse 3	KO 31	50	0	0 ± 0
	mouse 4	WT	45	100	443 ± 33
	mouse 5	WT	29	97	246 ± 28
	mouse 6	WT	50	98	219 ± 21
2	mouse 1	KO 31	50	0	0 ± 0
	mouse 2	KO 31	50	0	0 ± 0
	mouse 3	KO 31	55	0	0 ± 0
	mouse 4	WT	50	98	228 ± 20
	mouse 5	WT	50	91	195 ± 19
	mouse 6	WT	50	100	256 ± 16
3	feeder 1+3+5	KO 31	50	0	0 ± 0
	feeder 2+4+6	WT	50	100	33 ± 3.3
4	mouse 1	KO 45	39	0	0 ± 0
	mouse 2	KO 45	30	0	0 ± 0
	mouse 3	KO 45	30	0	0 ± 0
	mouse 4	WT	42	100	158 ± 14
	mouse 5	WT	30	100	154 ± 20
	mouse 6	WT	30	100	77 ± 13
5	feeder 1+3+5	KO 45	50	0	0 ± 0
	feeder 2+4+6	WT	50	78	134 ± 13

[a]Gametocyte feeds (mouse) or ookinete feeds (feeder).

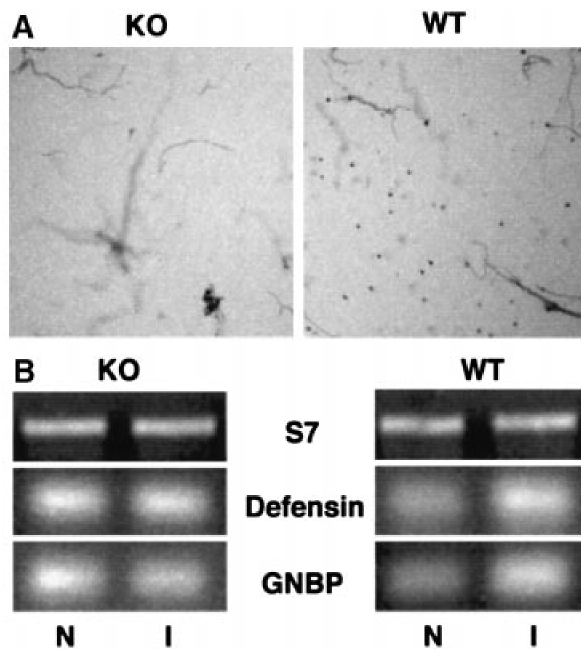

Fig. 3. Melanization of parasites and induction of an immune response in *A.gambiae* midguts. (**A**) Parts of refractory strain L 3-5 posterior midguts at 6 days after infection with WT or KO parasites. Black dots are encapsulated parasites. (**B**) RT–PCR expression analysis of the immune markers defensin and GNBP in naive (N) or infected (I) mosquitoes with WT or KO parasite. cDNA templates were normalized using the ribosomal protein S7 gene primers.

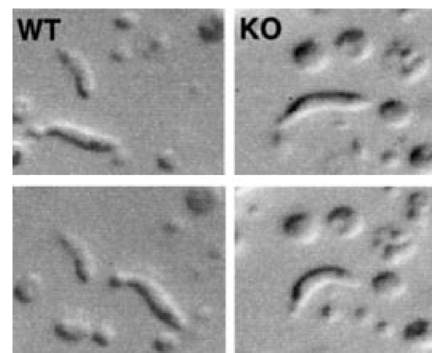

Fig. 4. Locomotion of WT and KO ookinetes. The left-hand side shows photographs of a typical WT ookinete taken 5 min apart, showing displacement. The right-hand side shows photographs of a typical KO ookinete taken 30 min apart, showing bending and straightening.

(Spaccapelo *et al*., 1997; Sultan *et al*., 1997), but failed to detect trails of ookinete-reactive material on glass surfaces. This may be because ookinetes are less motile than sporozoites. However, when we assessed ookinete motility by recording their positions on glass surfaces at 5 min time intervals, we observed displacement of WT but not KO ookinetes (Figure 4). These findings indicate that CTRP is involved in ookinete locomotion. The KO ookinetes were occasionally seen to straighten and bend (Figure 4), suggesting that this form of movement does not require CTRP. The latter observation is consistent with that of *TRAP* gene knockout sporozoites (Sultan *et al*., 1997).

Discussion

In this paper we show that CTRP is expressed in the ookinete and is targeted to micronemes. As such, CTRP is the first micronemal protein of the malaria ookinete identified. The structural features of CTRP, combined with its micronemal localization, are indicative of a role of the molecule in ookinete invasion of the mosquito midgut epithelium, which is a crucial step in the malaria transmission cycle. We have addressed the function of CTRP using new transgene technology developed for malaria parasites (van Dijk *et al*., 1995). Results obtained from transmission experiments with transgenic parasites containing a dis-

rupted *CTRP* gene demonstrate that CTRP has a vital role in ookinete to oocyst development *in vivo*.

We further show that the KO ookinetes do not invade the midgut epithelium. Ookinetes were not detected either within the epithelium or on the haemocoelomic surface of the midgut epithelium following infection with KO parasites. Moreover, the innate immune response in the infected mosquito, which is triggered by invasion of the midgut by the ookinete (Dimopoulos *et al*., 1997), was not elicited. Thus, CTRP is the first ookinete molecule shown to play a vital role in invasion, identifying it as a potential new target for malaria-transmission-blocking strategies. Based on published evidence that oocyst formation *in vivo* requires contact with the basal lamina on the haemolymph side of the stomach wall (Weathersby, 1952), the failure of the KO ookinete to reach the basal lamina is the most likely explanation for the observed lack of oocyst development.

Why does the KO ookinete not invade the midgut epithelium? Among the other members of the thrombospondin-related family of apicomplexan proteins, a role in parasite locomotion and invasion has also been attributed to the well-studied *Plasmodium* TRAP. This molecule is localized in the micronemes and to a lesser extent on the surface of salivary gland sporozoites (Rogers *et al*., 1992). Recombinant TRAP from the human parasite *P.falciparum* can bind to host ligands like sulfated glycosaminoglycans, to a human hepatocyte-derived cell line (HepG2) and to the basolateral cell membrane of human hepatocytes in the space of Disse (Müller *et al*., 1993; Robson *et al*., 1995). The adhesive properties of TRAP may be functionally linked to the process of substrate-dependent motility of the sporozoite: *P.berghei* sporozoites shed a continuous trail of TRAP-containing material during gliding on microscope slides and antibodies against TRAP dramatically block parasite motility (Spaccapelo *et al*., 1997). Moreover, transgenic sporozoites in which the *TRAP* gene is knocked out are impaired in gliding motility, but also in salivary gland invasion and infectivity to mice (Sultan *et al*., 1997; Wengelnik *et al*., 1999). More recent data show that transgenic sporozoites with a mutated type A domain in TRAP are impaired in salivary gland invasion but not gliding motility. This indicates that the molecule is also involved in recognition of or attachment to salivary glands, and that this process is functionally distinct from gliding motility (Wengelnik *et al*., 1999).

Our findings indicate that CTRP plays a role in ookinete locomotion. The impaired locomotion may prevent the KO ookinete from reaching and/or penetrating the target epithelial cells (Sibley *et al*., 1998). Alternatively, by analogy to *TRAP* knockout sporozoites and salivary glands, the KO ookinete may be unable to recognize or attach to the midgut epithelial cells. Both possibilities could explain the observed lack of invasion and oocyst development by these parasites. A dual function of CTRP in motility and binding is also possible, as is the case for TRAP. Although we did not detect CTRP on the ookinete surface it may be present in a small quantity or be translocated to the cell surface, possibly in response to stimuli encountered in the midgut. Here, CTRP could function by connecting the parasite actin–myosin motor with external substrates and/or by delivering a specific signal upon binding to host ligands, as has been proposed for TRAP (Sultan *et al*., 1997). The difference in the structures of CTRP and TRAP, most notably the multitude of adhesive domains (13 in CTRP and two in TRAP), may reflect differences in the substrates/ligands encountered by ookinete and sporozoite, respectively, or in the affinities of interactions required for their biological function. The involvement of *P.berghei* CTRP in motility and infectivity of the ookinete, combined with the knowledge that a structural homologue exists in human malaria, provides a rationale for the development of specific molecular inhibitors of ookinete locomotion as a new antimalarial strategy.

Materials and methods

Parasite maintenance, culture and purification

Plasmodium berghei ANKA parasites (clone 234) were used throughout this study except for the preparation of asexual blood-stages, for which the non-gametocyte-producing clone 233 was used. Parasites were maintained as cryopreserved stabilates or by mechanical blood passage and regular transmission through mosquitoes. Midgut-stage parasites were obtained by *in vitro* culture of gametocytes, allowing gamete, zygote and ookinete development (Sinden *et al*., 1985). For the time course, blood from different mice was pooled and white blood cells were removed with Plasmodipur filters (Eurodiagnostica, Arnhem, The Netherlands). The culture was split into 13 different culture flasks and placed at 20°C. Every 2 h (starting with time point zero) the blood from a flask was spun down, the red blood cells lysed using 0.17 M ammonium chloride and parasites pelleted. The pellet was frozen at –20°C until use. To obtain ookinetes or gametocytes free from asexual blood-stage parasites, infected mice were pyrimethamine treated (10 mg/kg) for 3 consecutive days before parasite harvest. White blood cells were removed on CF11 cellulose columns, and red blood cells lysed in 0.17 M ammonium chloride. Midgut-stage parasites were further purified on Nycodenz (Nycomed, Oslo, Norway) cushions (19% w/v) in phosphate-buffered saline (PBS). All purifications were performed at 4°C.

cDNA library construction

Subtracted cDNA was generated with the PCR-Select cDNA subtraction kit (Clontech) according to the manufacturer's instructions using midgut-stage parasites as tester and asexual blood-stage parasites (clone 233) as driver samples. Total RNA was purified from parasites using RNeasy columns (Qiagen) and mRNA was subsequently purified using Oligotex (Qiagen). PCR-amplified subtracted cDNA was ligated into pGEM-T Easy (Promega) and transformed into *Escherichia coli* JM109 cells. Abundant clones were selected with the PCR-Select differential screening kit (Clontech) according to the manufacturer's instructions and analysed by automated fluorescent sequencing.

Construction of transgenic parasites

The *Tg*DHFR/TM^R cassette was excised from p$\Delta D_B D_{TM} D_B$ (Waters *et al*., 1997) with *Hin*dIII and *Eco*RV, and cloned into *Hin*dIII–*Eco*RV-digested pBluescript SK, to give pBS-DHFR. A partial, 1080 bp cDNA corresponding to nucleotides 4399–5479 of *CTRP* (clone 2) was identified from a subtraction cDNA library. From this, a 450 bp sequence was PCR amplified with primers CTRP-Kpn (5′-GGGGTACCTTGTTCTT-CACAAGTTAAACCA-3′) and CTRP-Cla (5′-CCATCGATAAATATA-GTTGGCTTTAATGAAG-3′), *Kpn*I–*Cla*I digested and cloned into *Kpn*I–*Cla*I-digested pBS-DHFR, to give pCTRP-KC. An adjacent 530 bp sequence was PCR amplified from clone 2 with primers CTRP-Bam (5′-CGGGATCCGTAATAAAATGCCTTATTCTCAT-3′) and CTRP-Eag (5′-CCCGGCCGACAATGAAATGTGCCAAACA-3′), *Bam*HI–*Eag*I digested and cloned into *Bam*HI–*Not*I-digested pCTRP-KC, to give pCTRP-KO. Fifty micrograms of pCTRP-KO were digested with *Kpn*I and *Sac*II to excise the plasmid backbone and transfected into purified schizonts as described previously (Van Dijk *et al*., 1995; Waters *et al*., 1997). Pyrimethamine selection of transformed parasites and subsequent dilution cloning were as previously described (Waters *et al*., 1997).

Southern and Northern blotting

For Southern blotting, genomic DNA was extracted from purified blood-stage parasites by standard methods (Sambrook *et al*., 1989), digested with *Sph*I, fractionated in 0.8% agarose gels, transferred to Hybond N (Amersham) and hybridized overnight at 68°C by standard methods

(Sambrook *et al*., 1989). For Northern blotting, total RNA was extracted from parasites with RNeasy columns (Qiagen), fractionated in MOPS/formaldehyde-containing 1% agarose gels, transferred to Hybond N and hybridized overnight at 50°C in 50% formamide by standard methods (Sambrook *et al*., 1989). Washes were performed at high stringency (0.1× SSC). Probe was labelled with [^{32}P]dATP by standard random primer labelling (Sambrook *et al*., 1989).

Monoclonal antibody production

Myeloma cells (Pai) were fused with spleen cells from a Balb/c mouse immunized with *P.berghei* double gene knockout ookinetes that lack expression of Pbs21 and Pbs25 (kindly provided by C.J.Janse and A.P.Waters, University of Leiden, Leiden, The Netherlands). A cell line was selected that produced antibodies recognizing a protein of M_r ~200 000, and was cloned three times to give mAb 3A5. The antibody was shown to recognize CTRP specifically by Western analysis of CTRP KO parasites (Figure 2C).

Mosquito transmission assays

Anopheles mosquitoes were reared at 28°C and 75% humidity under a 12 h light/dark cycle and maintained on a 10% fructose solution. Adult female *A.stephensi* mosquitoes were starved overnight before blood feeding for 20 min. Parasites were used that were asexually passaged less than eight times (P8), the useful limit for mechanical transmission of clone 234 (Dearsly *et al*., 1990). Gametocyte feeds were performed by feeding mosquitoes directly on anaesthetized mice 4 days post-infection with *P.berghei* (5×10^7 parasites) as described (Sinden, 1997). Ookinete feeds were performed by feeding mosquitoes on membrane feeders containing 800 ookinetes/μl. Unfed mosquitoes were removed after feeding and the remaining mosquitoes were kept at 20°C with 10% fructose. Oocysts were counted at 10 days post-infection and transmission was expressed as the mean number of oocysts ± SEM.

Immunodetection of CTRP

Purified parasites were boiled for 5 min in non-reducing buffer and proteins fractionated by 4–20% gradient SDS–PAGE before Western blot analysis by standard methods (Sambrook *et al*., 1989). For immuno-fluorescent antibody staining, air-dried parasites on microslides were fixed in 1% formaldehyde and blocked in PBS plus 1% bovine serum albumin. After incubation for 1 h with mAb 3A5 (undiluted culture supernatant), slides were washed three times in PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody in PBS for 1 h and again washed three times in PBS. Slides were mounted in PermaFluor (Lipshaw Immunon, Pittsburgh, PA) and examined by UV/light microscopy. Immunogold electron microscopy was performed as described previously (Bannister and Kent,1993).

Invasion assays

Anopheles gambiae susceptible strain 4A r/r midguts were dissected at 24 h post-infection and fixed in 4% paraformaldehyde (in PBS) for 30 min. After removal of the blood meal, guts were fixed again for 30 min, washed four times in PBS and twice in PBS plus 0.1% Triton X-100, and incubated with mAb 13.1 (recognizing the ookinete surface antigen Pbs21) in PBS, Triton X-100 and 2% bovine serum albumin overnight at room temperature. After washing, guts were incubated with FITC-conjugated goat anti-mouse antibody for 2 h, washed, and examined by UV/light microscopy. *Anopheles stephensi* midguts at 24 h post-infection were left intact after dissection and were live stained with the FITC-conjugated antibody 12.1 (recognizing the ookinete surface antigen Pbs21) plus 0.1% (v/v) Hoechst 33258 (a permeant nuclear stain). Midguts were covered with vaseline slides so as not to compress and damage the gut, and examined by UV/light microscopy. This allowed simultaneous visualization of parasites and midgut epithelial cells. *Anopheles gambiae* refractory strain L 3-5 midguts were dissected at 6 days post-infection and fixed for 30 min in 4% paraformaldehyde in PBS before photography.

Expression analysis of immune markers by RT–PCR

RT–PCR expression analysis of immune markers in whole mosquitoes was performed as previously described (Dimopoulos *et al*., 1997). PCR cycles were 23 for S7, 31 for GNBP and 26 for defensin.

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