*Brief Definitive Report*

# Targeted Disruption of the *Plasmodium berghei* CTRP Gene Reveals Its Essential Role in Malaria Infection of the Vector Mosquito

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## Summary

CTRP (circumsporozoite protein and thrombospondin-related adhesive protein [TRAP]-related protein) of the rodent malaria parasite *Plasmodium berghei* (PbCTRP) makes up a protein family together with other apicomplexan proteins that are specifically expressed in the host-invasive stage (1). PbCTRP is produced in the mosquito-invasive, or ookinete, stage and is a protein candidate for a role in ookinete adhesion and invasion of the mosquito midgut epithelium. To demonstrate involvement of PbCTRP in the infection of the vector, we performed targeting disruption experiments with this gene. *PbCTRP* disruptants showed normal exflagellation rates and development into ookinetes. However, no oocyst formation was observed in the midgut after ingestion of these parasites, suggesting complete loss of their invasion ability. On the other hand, when ingested together with wild-type parasites, disruptants were able to infect mosquitoes, indicating that the PbCTRP gene of the wild-type parasite rescued infectivity of disruptants when they heterologously mated in the mosquito midgut lumen. Our results show that PbCTRP plays a crucial role in malaria infection of the mosquito midgut and suggest that similar molecular mechanisms are used by malaria parasites to invade cells in the insect vector and the mammalian host.

Key words: malaria • *Plasmodium berghei* • ookinete • midgut • gene targeting

After gamete fertilization and zygote formation in the midgut lumen of the Anopheline mosquito, malaria parasites transform into the invasive form, ookinete. Mature ookinetes attach to the surface of the midgut epithelium, migrate through it, and arrive at the basement membrane, where they stop moving and develop into oocysts. Presumably, molecular interactions between ookinetes and the midgut epithelium play an important role in this invasive process. However, no molecule that may mediate this interaction has been identified.

The circumsporozoite protein and TRAP (thrombospondin-related adhesive protein)-related protein (CTRP) gene has been cloned from the genome of the human malaria parasite *Plasmodium falciparum*, but its expression stage and function remains unclear (2). The CTRP gene encodes a 2,098–amino acid protein with a single transmembrane protein–like structure. The putative large extracellular region is composed of six integrin I region–like domains and seven thrombospondin-like domains. This structure is similar to that of other apicomplexan proteins such as TRAP, a malaria sporozoite protein, and micronemal protein 2, a tachyzoite protein of *Toxoplasma gondii* (3–4). These proteins are specifically produced in the host-invasive stages and thought to be critical for motility and invasion into host cells (5–6). In fact, it has been reported that disruption of the TRAP gene resulted in severe reduction of malaria sporozoite motility and infectivity (7). Recently, we found that CTRP of the rodent malaria parasite *Plasmodium berghei* (PbCTRP) is expressed in the ookinete (1). PbCTRP is produced at least 10 h after fertilization, when zygotes begin transformation into ookinetes. It is actively produced during ookinete development and ultimately observed in the anterior cytoplasm of mature ookinetes. This expression profile and its structure, described above, strongly indicate that PbCTRP plays a role in ookinete invasion into the mosquito midgut epithelium.

The purpose of this study is to demonstrate this possible role of PbCTRP. We performed targeting disruption experiment with this gene. The results show that *PbCTRP*-disrupted parasites are not able to infect the vector. This indicates that PbCTRP may mediate active invasion of the ookinete into the mosquito midgut epithelium.

## Materials and Methods

*Selectable Marker.* The wild-type dihydrofolate reductase thymidylate synthase gene of *P. berghei* with a 2.2-kb upstream and 0.75-kb downstream region was cloned from the genomic library and subcloned into a plasmid vector, pBluescript II. Resistance to pyrimethamine was conferred to this gene by a single amino acid

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/12/1711/05 $5.00
Volume 190, Number 11, December 6, 1999 1711–1715
http://www.jem.org

mutation ($Ser^{110} \rightarrow Asp^{110}$) using PCR (8). The validity of this gene as a selectable marker was confirmed by transformation of parasites with this plasmid and subsequent selection by pyrimethamine as previously described (9).

*Targeting Vector*. The DNA fragment containing the 5′ portion of *PbCTRP* (2.05 kb) was subcloned into pBluescript II. The selectable marker gene was inserted into the MunI site of this fragment after ligation of EcoRI linkers to both ends. For the gene targeting experiment, the plasmid was completely digested with restriction enzymes XhoI and NotI to separate the linear targeting construct from the plasmid backbone.

*Gene Targeting Procedure*. The gene targeting experiment was performed following the essentially same procedure as described by Menard et al. (10). In brief, merozoites of *P. berghei* were transfected by electroporation with 40 μg of linearized targeting vector, injected intravenously into a rat, and selected by pyrimethamine. The selected parasites were further separated into the wild-type parasite population and disruptants by limiting dilution. The infected parasite population of each rat was determined by PCR and Southern blot analysis.

*Southern Blot Analysis*. Southern blot analysis was performed as previously described (1). In brief, genomic DNA of the parasites was digested with restriction enzyme MunI, separated on a 0.7% agarose gel, and transferred to a nylon membrane. The blot was hybridized with a [$^{32}$P]dCTP-labeled HindIII/MunI-digested DNA fragment (0.8 kb) of *PbCTRP*. In rescue experiments, Southern blot analyses were performed with the same procedure. Radioactivity of the 2.5- and 6.5-kb bands was measured by the BAS 2000 system (Fuji Photo Film Co.), and the ratio of *CTRP* disruptants to wild-type parasites was estimated.

*Infection of Mosquitoes*. After checking the number of exflagellated parasites in the infected blood (>50 per $10^5$ erythrocytes), rats were subjected to bites of *Anopheles stephensi* mosquitoes for 30 min. The engorged mosquitoes were selected and maintained at 20°C. These mosquitoes were dissected 12 d after feeding, and oocysts in their midguts were carefully counted under a microscope with magnifications of 100 and 200.

## Results and Discussion

*Targeting Disruption of the PbCTRP Gene*. Fig. 1 a shows the targeting construct used in this experiment. It is composed of a selectable marker that confers pyrimethamine (antimalarial drug) resistance to parasites and *CTRP* sequences ligated at both ends. Merozoites of *P. berghei* were transfected with this construct by electroporation and intravenously injected into a naive rat. Integration of this construct into the *CTRP* locus by homologous recombinations resulted in disruption of this single-copy gene. The CTRP gene–disrupted parasites were selected in the rat by pyrimethamine. PCR and Southern blot analysis showed that the parasites selected with pyrimethamine were a mixture of wild-type parasites and *CTRP* disruptants (Fig. 1, b and c, selected). They were separated by limiting dilution and subsequent inoculation into a group of 20 rats. Out of 12 infected rats, 7 were infected only by *CTRP* disruptants and 4 were infected only by wild-type parasites. In these parasites, 4 disruptants and 3 wild-type parasite populations were used in the experiments described below (Fig. 1, b and c).

*Parasite Infectivity Was Completely Lost by Disruption of the PbCTRP Gene*. All seven parasite populations developed

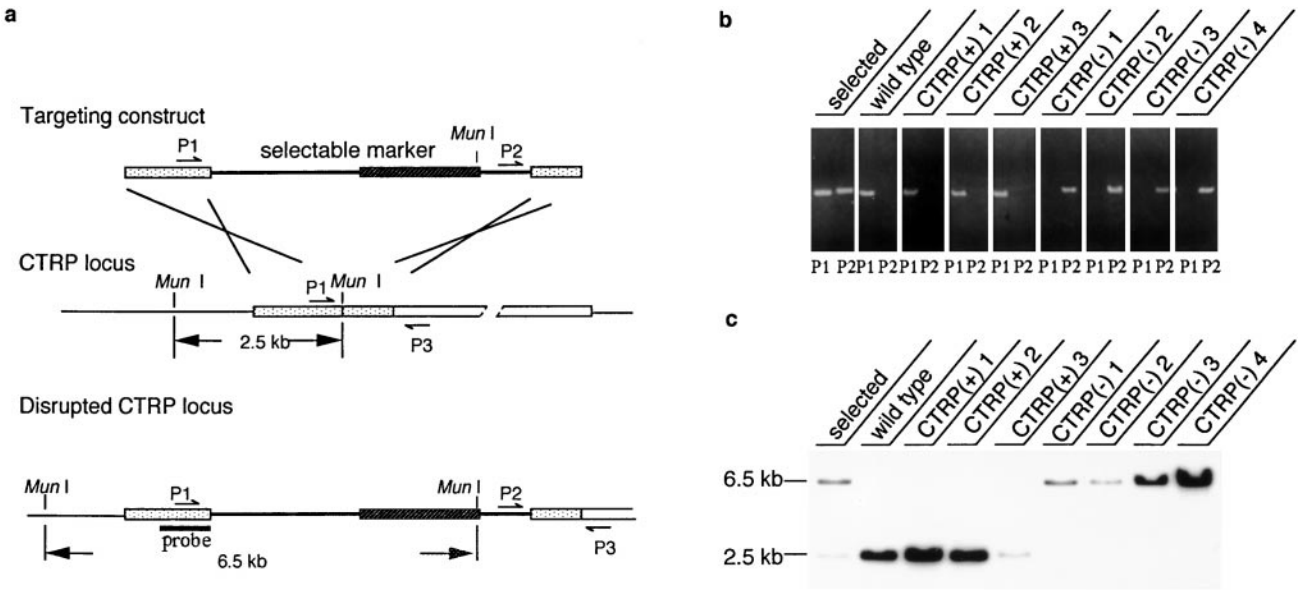


**Figure 1.** Targeted disruption of the CTRP gene in *P. berghei*. (a) Targeting strategy. The targeting vector (top line) was integrated into the CTRP locus (center line) by double cross-over between CTRP sequences of the targeting vector and corresponding sites in the CTRP locus, which results in disruption of the CTRP gene locus (bottom line) and confers pyrimethamine resistance to the CTRP gene–disrupted parasites. Primers (P1, P2, and P3) used for PCR analysis in b and the probe used for Southern blot analysis in c are indicated. (b) PCR analysis of parasites includes parasites selected by pyrimethamine (selected), wild-type parasites as control (wild-type), and three wild-type (CTRP(+) 1–3) and four CTRP disruptant (CTRP(−) 1–4) populations. PCR with primers P1 and P3 (left lanes, P1) gave an amplified 900-bp product from wild-type parasites, whereas PCR with primers P2 and P3 (right lanes, P2) resulted in a 950-bp product from disruptants. (c) Southern blot analysis of the same parasite populations as in b. DNAs isolated from the respective parasite populations were hybridized with the indicated probes after MunI digestion.

into mature ookinetes in vitro within 20 h. These ookinetes did not show any morphological differences from wild-type parasites with Giemsa staining under microscopic observation (Fig. 2 a). Disruption of *CTRP* loci was further confirmed by immunocytochemistry (Fig. 2 b). The infected rats were separately subjected to bites of *Anopheles stephensi* mosquitoes to assess the ability of these parasite populations to infect the insect vector. Before these mosquito challenges, all seven parasite populations showed normal exflagellation numbers in vitro (>50 exflagellations per $10^5$ erythrocytes). Mosquitoes were dissected 12 d after feeding, and the number of oocysts in their midguts was counted (Table I). In the wild-type populations, a total of 41 out of 48 mosquitoes was infected, and a total of 666 oocysts was found in the mosquito midguts. All infected mosquitoes had at least one oocyst containing clearly differentiated sporozoites. In contrast, no oocysts were found in 120 mosquitoes fed on the rats infected with *CTRP*-disruptant populations.

We also examined the mortality rate of blood-fed mosquitoes (until day 12) in every parasite population. The mortality rates in the wild-type parasite populations (CTRP(+) 1–3) were 32.0, 33.3, and 50,0%, respectively. On the other hand, those in disruptants (CTRP(−) 1–4) were 18.8, 3.5, 20.0, and 9.5%, respectively. In total, the mortality rate of the mosquitoes in the *CTRP* disruptants was 13.9% (33 out of 237 mosquitoes), and the mortality rate in wild-type parasite populations was 36.7% (68 out of 185 mosquitoes). It has been reported that mosquito mortality rate increases after *P. berghei* infection by the damage of the midgut barrier by parasite penetration and after bacterial infection (11). Therefore, the difference in mosquito mortality rate between wild type and disruptant might indicate that *CTRP*-disrupted ookinetes could not penetrate the midgut epithelium.

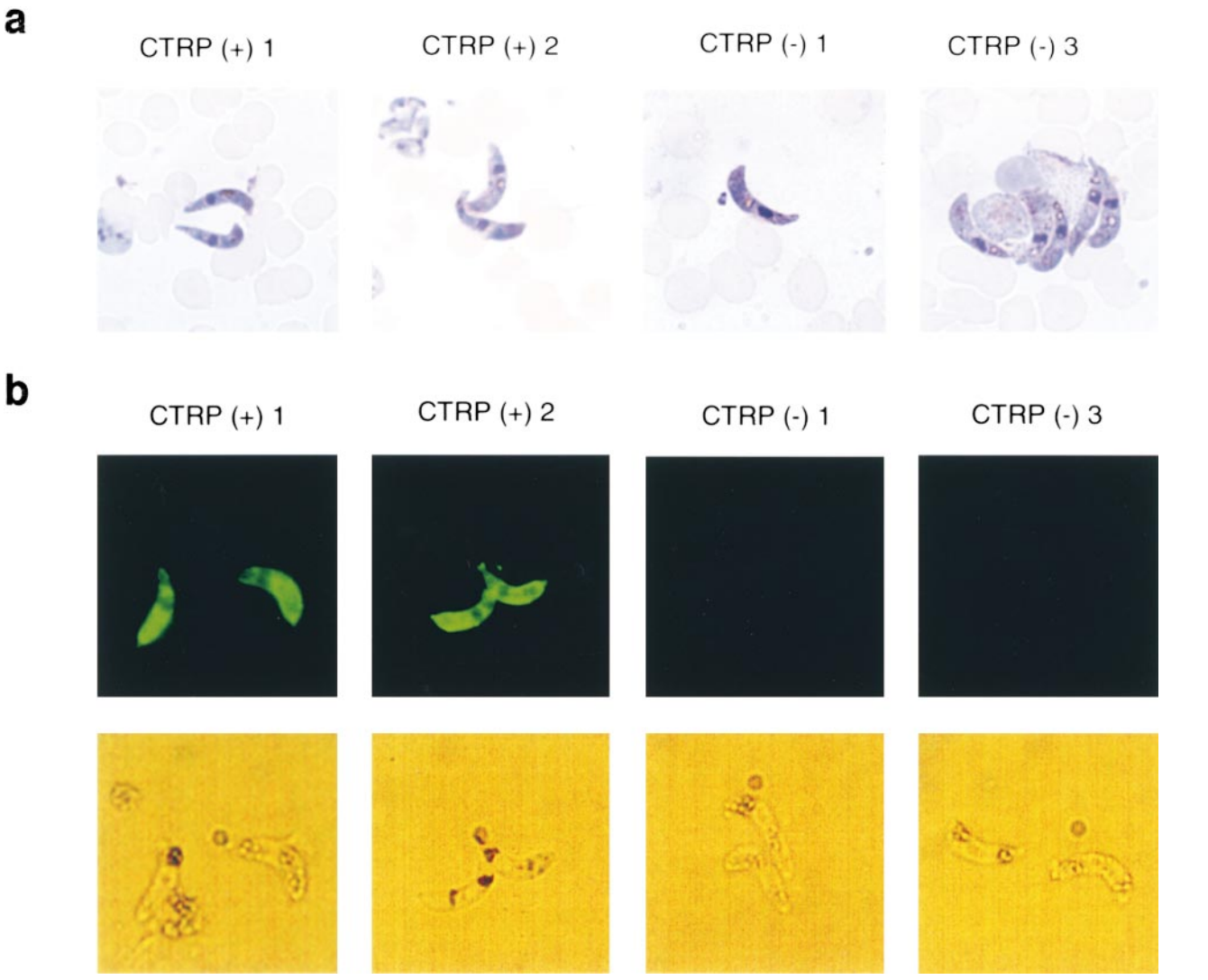


**Figure 2.** Comparison of CTRP (+) and (−) populations by Giemsa staining and immunocytochemistry. (a) Optical microscopy of the ookinetes of wild-type (CTRP (+) 1 and CTRP (+) 2) and CTRP-disruptant (CTRP (−) 1 and CTRP (−) 3) populations. The infected rat blood was cultured in the ookinete culture medium for 20 h, and the blood smear was stained with Giemsa. Magnifications 400. (b) Expression of CTRP in the ookinetes of the same parasite populations. Top panels, ookinetes cultured for 20 h were purified by erythrocyte lysis and stained with primary antibody and FITC-conjugated secondary antibody. Bottom panels, translucent views. Magnifications 400.

**Table I.** *CTRP Disruptants Lost Their Infectivity*

| Parasite population | Dissected | Infected (%) | Mean no. oocysts* | Oocysts (total)/ Mosquitoes (total)‡ |
|---|---|---|---|---|
| CTRP (+) 1 | 23 | 21 (91) | 19 | |
| CTRP (+) 2 | 6 | 5 (83) | 9 | |
| CTRP (+) 3 | 19 | 15 (79) | 15 | 666/48 |
| CTRP (−) 1 | 30 | 0 (0) | – | |
| CTRP (−) 2 | 30 | 0 (0) | – | |
| CTRP (−) 3 | 30 | 0 (0) | – | |
| CTRP (−) 4 | 30 | 0 (0) | – | 0/120 |

Mosquitoes were dissected 12 d after feeding and examined for oocysts in the midgut under a microscope.
*Number of oocysts/number of infected mosquitoes.
‡Number of total oocysts/number of dissected mosquitoes.

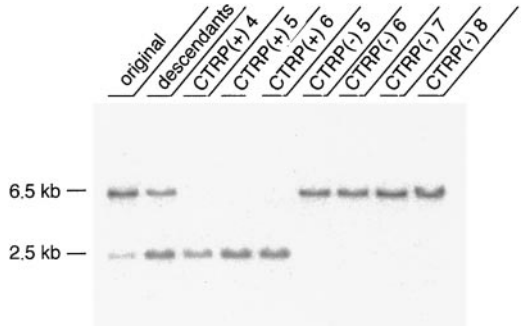


**Figure 3.** CTRP disruptants were rescued by wild-type parasites. A mixture of disruptants and wild-type parasites (original) was used to infect mosquitoes. Two naive rats were infected from these mosquitoes, and intraerythrocytic stage parasites were collected 6 d after mosquito bites. Parasites in one of these rats (descendants) were further separated into wild-type (CTRP(+) 4–6) and CTRP-disruptant populations (CTRP(−) 5–8) by limiting dilution. Southern blot analysis was performed using the same probe as in Fig. 1 c. The ratio of CTRP disruptants to wild-type parasites was estimated based on radioactivity of 2.5- and 6.5-kb bands as measured by the BAS 2000 system (Fuji Photo Film Co.).

*Mating with Wild-Type Parasite Rescued the Infectivity of PbCTRP Disruptants.* The malarial ookinete develops from the zygote after fertilization in the mosquito midgut lumen. This is the only stage in the life cycle of *Plasmodium* that is diploid with heterologous chromosomes. Assuming that the PbCTRP gene from wild-type parasites would compensate for a disrupted CTRP gene when they mated heterologously in the mosquito midgut lumen, we performed the following rescue experiment. We prepared a rat infected with both wild-type parasites and *CTRP* disruptants. The proportion of *CTRP* disruptants to wild-type parasites in this rat was estimated as 5.5:1 by Southern blot analysis (Fig. 3, original). Mosquitoes were infected by feeding on this rat. Parasites were fertilized, allowed to complete the sporogonic stage in these mosquitoes, and further transmitted to two other naive rats by bites from these mosquitoes (using 15 mosquitoes each). PCR and Southern blot analysis showed that these rats were infected with disruptants as well as wild-type parasites. The proportion of *CTRP* disruptants to wild-type parasites in these infected rats was estimated to be 0.68:1 (Fig. 3, descendants) and 0.58:1 by Southern blot analysis. These values are in good accordance with the figure calculated from the value in the original parasites, assuming that the heterozygous ookinete shows a normal phenotype (5.5:5.5 + 1 = 0.85:1). This indicates that these disruptants were rescued by mating with the wild-type parasites. This result also demonstrates that CTRP is not essential for other invasive stages, because fertilized parasites become haploid again after sporogony in the mosquito midgut.

We further separated these parasites (Fig. 3, descendants) into wild-type parasite populations and disruptants by limiting dilution (Fig. 3, CTRP(+) 4–6 and CTRP(−) 5–8) and then performed mosquito infection experiments using the same procedure as in Table I to confirm that the descended disruptants had really been rescued by mating with wild-type parasites. In the wild-type populations, a total of 1,951 oocysts was found in wild-type parasite populations (41 mosquitoes). However, no oocysts were found in the four *CTRP* disruptant populations (117 mosquitoes), indicating that rescued disruptants lost their infectivity again by separation from the wild-type parasites. The mortality rate was lower in those mosquitoes that fed on rats infected with *CTRP* disruptants (14.3%) compared with the wild-type parasite populations (30.2%).

*Conclusion.* In this study, we performed targeted disruption of the PbCTRP gene. Disruption of the CTRP gene resulted in complete loss of ookinete infectivity. However, it did not apparently influence ookinete maturation and morphology. In addition, rescue experiments demonstrate that PbCTRP is essential only in the diploid stage. These results are comparable to those previously reported in the targeted disruption experiment of the TRAP gene. Considering the structural similarity between CTRP and TRAP, these molecules may play a related role in the machinery of the respective stages of active invasion. Although the mechanism of malaria parasite invasion of the vector mosquito is poorly understood, further studies aimed at identification of other molecular components interacting with CTRP as part of this invasion machinery will enhance understanding of the parasite–vector interactions.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (08281103) and for Exploratory Research (10877043, 11877045) from the Ministry of Education, Science, Culture, and Sports of Japan and by a grant from the Research for the Future Program from the Japan Society for the Promotion of

Science to Y. Chinzei, and by a Grant-in-Aid (1998) from the Mie Medical Research Foundation to M. Yuda.

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*Submitted: 2 July 1999 Revised: 23 August 1999 Accepted: 26 August 1999*

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