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# The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands

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**Sporozoites from all *Plasmodium* species analysed so far express the thrombospondin-related adhesive protein (TRAP), which contains two distinct adhesive domains. These domains share sequence and structural homology with von Willebrand factor type A-domain and the type I repeat of human thrombospondin (TSP). Increasing experimental evidence indicates that the adhesive domains bind to vertebrate host ligands and that TRAP is involved, through an as yet unknown mechanism, in the process of sporozoite motility and invasion of both mosquito salivary gland and host hepatocytes. We have generated transgenic *P.berghei* parasites in which the endogenous *TRAP* gene has been replaced by either *P.falciparum TRAP* (*PfTRAP*) or mutated versions of *PfTRAP* carrying amino acid substitutions or deletions in the adhesive domains. *Plasmodium berghei* sporozoites carrying the *PfTRAP* gene develop normally, are motile, invade mosquito salivary glands and infect the vertebrate host. A substitution in a conserved residue of the A-domain or a deletion in the TSP motif of PfTRAP impairs the sporozoites' ability to invade mosquito salivary glands. Notably, midgut sporozoites from these transgenic parasites are still able to infect mice. Midgut sporozoites carrying a mutation in the A-domain of PfTRAP are motile, while no gliding motility could be detected in sporozoites with a TSP motif deletion.**

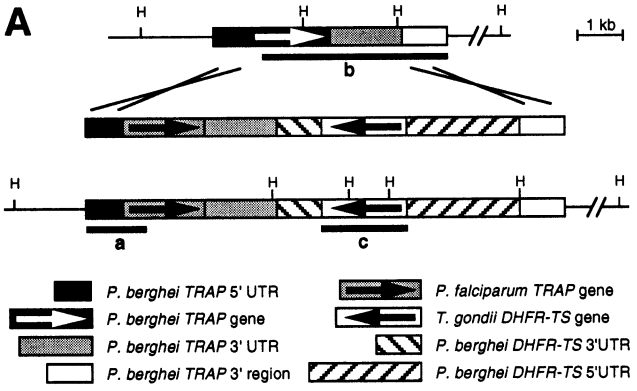
*Keywords*: gene complementation/gliding motility/malaria/TRAP

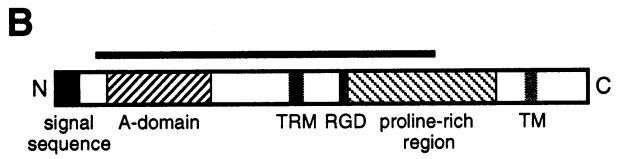
## Introduction

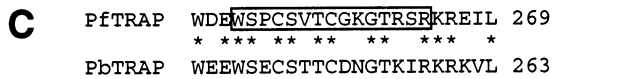
The thrombospondin-related adhesive protein of *Plasmodium falciparum* (PfTRAP) is encoded by a sporozoite-transcribed gene. PfTRAP homologues have been found in all *Plasmodium* species analysed so far, which share a similar overall structure (Robson *et al*., 1997, 1998; Templeton and Kaslow, 1997; Naitza *et al*., 1998) (see Figure 1B). The N-terminal region encompasses a 200-amino-acid sequence that shows structural homology with the von Willebrand factor type A-domain (Sixma *et al*., 1991). This sequence is clearly related to the inserted domain (I-domain) of the α chain of certain integrins, such as CD11b (Lee *et al*., 1995b). In TRAP, the A-domain is followed by a stretch of 40 amino acids encompassing a motif similar to the type I repeat of thrombospondin (TSP) (Lawler and Hynes, 1986). At the C-terminal end, all TRAP molecules have a highly conserved hydrophobic stretch of amino acids displaying the features of a transmembrane domain. This region is followed by a putative acidic cytoplasmic tail of ~43–45 amino acids. These structural features, together with the localization of TRAP within the micronemes and on the sporozoite surface (Rogers *et al*., 1992), suggest a role for this molecule in the process of parasite recognition of vertebrate host and/or arthropod vector ligands (Naitza *et al*., 1998).

Recombinant TRAP from the human parasite *P.falciparum* (PfTRAP) has been shown to bind to sulfated glycosaminoglycans (GAGs), 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 region of PfTRAP was mapped to a 60-amino-acid region that encompassed the TSP-related motif (TRM) (Müller *et al*., 1993). In contrast, the functional significance of the A-domain has not yet been clarified. By analogy with vertebrate integrin, this domain is anticipated to confer to TRAP the ability to interact with several host molecules. These possible ligands include collagen, the major complement C3 opsonin (iC3b) (Ueda *et al*., 1994), intercellular adhesive molecule 1 (ICAM-1) (Randi and Hogg, 1994), and the coagulation factors fibrinogen or factor X (Altieri and Edgington, 1988). Attempts have so far failed to show interaction of known A-domain ligands with malaria sporozoites and/or recombinant TRAP constructs. Moreover, *Plasmodium yoelii* sporozoites were shown to infect mice with targeted deletions of either the ICAM-1 or the complementary C3 genes (Sultan *et al*., 1997a).

Recent experimental evidence indicated that the adhesive properties of TRAP may be functionally linked to the process of substrate-dependent motility of malaria sporozoites. *Plasmodium berghei* sporozoites were shown to shed a continuous trail of TRAP-containing material during gliding motility on microscope slides, and antibodies against TRAP dramatically blocked parasite motility (Spaccapelo *et al*., 1997). Transgenic *P.berghei* sporozoites in which the *TRAP* gene had been disrupted were not motile, failed to invade mosquito salivary glands and showed a drastic reduction in their ability to infect host hepatocytes (Sultan *et al*., 1997b). It has been speculated that TRAP could function by connecting the parasite actin–myosin motor with external substrates and/or by delivering a specific signal upon binding to host ligands



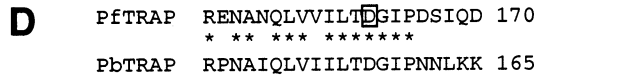




**Fig. 1.** (**A**) Schematic representation of the genomic *PbTRAP* locus (top line), the DNA construct used for gene targeting (middle line) and the genomic organization of the resulting transgenic *P.berghei* parasites. Three DNA fragments from the untranslated regions (UTR) 5′ and 3′ of the *PbTRAP* gene were cloned adjacent to the *PfTRAP* gene and the selectable marker, the pyrimethamine-resistant form of the *Toxoplasma gondii DHFR-TS* transcription unit. Double cross-over at the ends of the linear DNA construct with homologous genomic sequences leads to the deletion of the endogenous *PbTRAP* gene, its replacement by the *PfTRAP* gene and the insertion of the selectable marker. Arrows indicate the direction of transcription. The relative positions of the *Hinc*II sites (H) and the three probes (a, b, c) used in Southern blot analysis are indicated. (**B**) Diagram of PfTRAP and its structural features. The relative positions of the adhesive domains (A-domain, TRM and RGD motif), the signal sequence and the transmembrane (TM) domain as well as the proline-rich region are shown. The bar above the diagram indicates the sequences deleted in the TRAP knock-out. (**C**) Sequence alignment of the TRM from PfTRAP and PbTRAP. The boxed sequence has been deleted in the PfTRAP TRM mutant. (**D**) Sequence alignment of a part of the A-domain of PfTRAP and PbTRAP. Identical residues are marked by an asterisk. The labelled aspartate has been substituted by an alanine in the PfTRAP A-domain mutant.

(Sultan *et al*., 1997b). This hypothesis takes into account the microneme/surface localization of TRAP (Rogers *et al*., 1992), its adhesive properties for host ligands (Müller *et al*., 1993) as well as the phenotype of TRAP knock-out parasites (Sultan *et al*., 1997b). The individual function of the two adhesive domains of TRAP and their role in parasite motility, sporozoite recognition and invasion of salivary glands in the mosquito and of hepatocytes in the vertebrate host remain unexplained. In particular it is not clear whether the potential recognition of ligands by the A-domain and the TRM only provides attachment to substrates for sporozoite gliding, or if it also determines the specificity of malaria sporozoites for mosquito salivary glands and host hepatocytes.

To address these questions we have carried out a structure–function analysis of TRAP. We have developed transgenic *P.berghei* sporozoites in which the endogenous *TRAP* gene has been replaced by either wild-type *PfTRAP* or distinct versions of *PfTRAP* mutagenized in the A-domain and the TRM. These transgenic parasites have been analysed for their ability to invade mosquito salivary glands, infect mice and glide on glass surfaces.

## Results

### *Development of PbTRAP targeting vectors*

Although the development of gene transfer technology in *Plasmodium* parasites has offered a great opportunity to study gene function *in vivo*, its full potential is limited by the availability of one selectable marker. Transformed *P.berghei* can be selected only by using a mutated version of dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) that confers resistance to the antimalarial pyrimethamine (van Dijk *et al*., 1995). To overcome this limitation, constructs were designed to achieve deletion of the endogenous *PbTRAP* gene, insertion of wild-type or mutated *PfTRAP* versions, and the insertion of the selectable marker transcription unit in a single transformation event. The DNA was introduced into the genome by double cross-over homologous recombination. This approach has the advantage of being non-reversible, in contrast to gene targeting mediated by single homologous recombination (Sultan *et al*., 1997b), and overcomes the parasite repair mechanism. Small mutations within the homologous gene targeting sequences have recently been shown to be replaced by wild-type sequences in *P.berghei* (Nunes *et al*., 1999). Furthermore, the replacement of the endogenous *PbTRAP* gene with *PfTRAP* will facilitate the identification of transgenic parasites at any given stage of the life cycle by using either species-specific PCR primers or monoclonal antibodies. The transformation construct, which has been used to replace *PbTRAP* by *PfTRAP* (ITO 4 isolate), is represented schematically in Figure 1A. Untranslated regions (UTRs) 5′ and 3′ of the *PbTRAP*-coding sequence were cloned to flank the *PfTRAP* gene and the *DHFR-TS* transcription unit in the plasmid construct. The 5′ UTR spanning 0.8 kb immediately upstream of *PbTRAP* was inserted in front of the complete wild-type *PfTRAP* gene. Although the 5′ UTR has not been characterized in terms of promoter activity, recombination with the homologous region in the genome will place the *PfTRAP* gene under the control of all *PbTRAP* 5′ regulatory sequences. The contribution of the 3′ UTR to transcriptional regulation and stability of the transcript has not yet been elucidated in *Plasmodium*. To overcome any problems that might arise, we inserted a 1.6 kb DNA sequence from the *PbTRAP* 3′ UTR downstream of *PfTRAP*. The *DHFR-TS* selectable marker transcription unit was cloned in the inverse orientation to the transcriptional direction of the *TRAP* gene on the assumption that

this would avoid transcriptional interference of the two units. The *DHFR-TS* gene of *Toxoplasma gondii* was used as the selectable marker because it results in 100-fold higher resistance to pyrimethamine than *P.berghei DHFR-TS* (Waters *et al*., 1997). This gene is under transcriptional control of 5′ and 3′ UTRs from the *P.berghei DHFR-TS* locus. A fragment of 1 kb located 1.6 kb downstream of *PbTRAP* was cloned at the extremity of the construct to supply the DNA end for the second homologous recombination event. Double homologous recombination at the ends of this linear DNA construct will lead to: (i) the deletion of endogenous *PbTRAP*; (ii) its replacement by *PfTRAP* at a position identical to that of the wild-type genome and under the control of endogenous regulatory sequences; and (iii) the insertion of the selectable marker downstream of *PfTRAP* (Figure 1A). This construct was cloned in a pUC19 vector and named pTV-WT.

Three *TRAP* mutated constructs were generated using derivatives of the plasmid pTV-WT. Deletion of a 1090 bp *Bgl*II fragment within the *PfTRAP* gene (positions 141–1231: plasmid pTV-1) generated a truncated version of PfTRAP with a frameshift mutation after amino acid 48 (Figure 1B). This *PfTRAP Bgl*II fragment was also replaced with two mutated *PfTRAP* sequences. A 45 bp deletion in the TRM resulted in the loss of the most conserved portion of this adhesive domain (amino acids 250–264, WSPCSVTCGKGTRSR) (plasmid pTV-TRM) (Figure 1C). A second construct contained a single A to C point mutation at position 485 that led to the substitution of the highly conserved Asp162 residue by alanine (plasmid pTV-A) (Figure 1D). This mutation is predicted, by analogy to the effect of a similar mutation (D242A) in the A-domain of human β2 integrin CD11b, to disrupt the correct folding of TRAP. X-ray crystallography of CD11b has shown that this aspartate is a crucial residue of the metal-ion-dependent adhesion site (MIDAS) of the A-domain (Lee *et al*., 1995a,b). The D242A mutation resulted in a loss of binding to divalent cations as well as to the ligand opsonin iC3b (Michishita *et al*., 1993).

By using the constructs pTV-WT, pTV-1, pTV-A and pTV-TRM in gene targeting experiments on *P.berghei* schizonts, we have generated the transgenic parasite populations 87 (wild-type PfTRAP replacement), L3 (PbTRAP knock out), L5 (PfTRAP A-domain mutation), and L8 (PfTRAP TRM deletion), respectively.

### *Molecular analysis of the transgenic parasites*

Transgenic parasites were selected *in vivo* by their resistance to pyrimethamine, and cloned by limiting dilution. Clones 87-11, L3-19, L5-13 and L8-9 were used for all further studies. Southern blot analysis demonstrated that double homologous recombination had been achieved as expected in all four transgenic clones. Probe a, encompassing the *PbTRAP* 5′ UTR and part of the *PfTRAP* sequence (Figure 1) showed, on a *Hinc*II digest of total DNA, the correct integration event at the left end of the construct (Figure 2). A digestion fragment of 3.5 kb present in the wild-type DNA increased in size in the transgenic parasites to 4.5 kb in the knock-out and to 5.6 kb in the other three mutants, respectively. This change in electrophoretic mobility was due to the presence of a *Hinc*II site within *PbTRAP* which was absent in *PfTRAP*. The integration of the right-hand end of the construct was

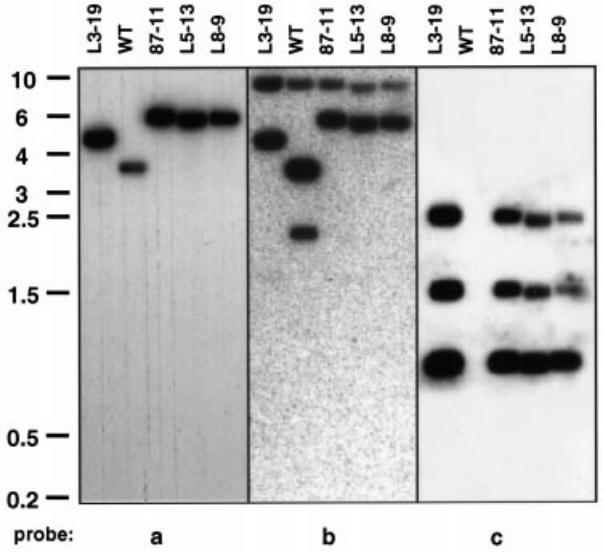



**Fig. 2.** Southern blot analysis of transgenic clones. Total genomic DNAs of *P.berghei* wild type (WT), the TRAP knock-out clone L3-19, the PfTRAP replacement clone 87-11, the PfTRAP A-domain mutant clone L5-13 and the PfTRAP TRM deletion clone L8-9 were digested with *Hinc*II, separated on a 1% agarose gel and blotted onto nylon membrane. The same membrane was consecutively hybridized with a probe from the *PbTRAP* 5′ UTR (a), the *PbTRAP* gene and its 3′ UTR (b) and the *T.gondii DHFR-TS* gene (c) (see Figure 1). DNA marker sizes are given in kilobases on the left.

demonstrated using probe b, encompassing the majority of the *PbTRAP* sequence and 2.8 kb of its 3′ UTR (Figure 1). This probe hybridized to the same digestion fragments as in sequence a together with a 2.2 kb fragment originating from the 3′ sequences of *PbTRAP* in the wild-type DNA (Figure 2). The presence of the same 9 kb fragment in all five clones indicated that the region to the right of the recombination event has not been rearranged. By using the *T.gondii DHFR-TS* gene as probe c, we demonstrated that the selectable marker had been correctly maintained after insertion in the four transgenic clones and was absent from the wild-type parasite DNA. PCR analysis using primer pairs specific for *PbTRAP* or *PfTRAP* was used throughout this study as a rapid and sensitive assay for monitoring the transformed parasite populations and the selected clones. Restriction polymorphisms in the *PfTRAP* constructs were used to differentiate between the mutants, e.g. gain of a *Pvu*II site in the A-domain mutant, or loss of a *Kpn*I site in the TRM mutant (data not shown).

### *Life cycle of the transgenic parasites: PfTRAP complements for PbTRAP function*

We did not observe differences in blood-stage replication and the development of gametocytes when comparing wild-type parasites with the transgenic clones. Exflagellation of male gametes and fertilization occurred in transgenic parasites with kinetics similar to the wild-type parental strain, giving rise to morphologically normal ookinetes in ookinete culture medium (data not shown). Naive *Anopheles stephensi* mosquitoes were fed on mice infected with the PfTRAP replacement clone 87-11 and the development of the parasites was monitored. Oocysts developed comparably to *P.berghei* wild type. At day 14 post-infection (p.i.), ~30% of the oocysts from both clones contained sporozoites. Salivary glands were dissected and

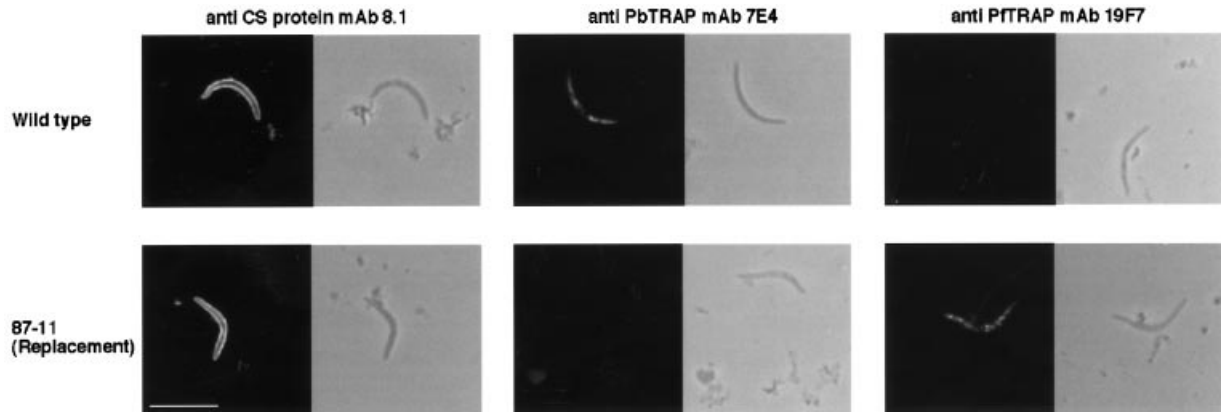



**Fig. 3.** Confocal fluorescence (dark field) and transmission microphotographs (bright field) of *P.berghei* sporozoites. Wild-type and 87-11 salivary gland sporozoites were stained with mAb 8.1, 7E4 and 19F7 directed against *P.berghei* CS protein, PbTRAP and PfTRAP, respectively. Magnification ×630, plus zoom factor 2.0 for image acquisition. Scale bar = 10 μm.

homogenized at day 21 p.i. Salivary gland sporozoites were analysed for the expression of PfTRAP by immunofluorescence (IF) using the monoclonal antibodies (mAb) 19F7 (Müller *et al*., 1993) and 7E4 directed against PfTRAP and PbTRAP, respectively. The mAb 19F7, raised against bacterial recombinant PfTRAP, has previously been used to analyse the expression and the localization of PfTRAP during sporozoite ontogeny (Müller *et al*., 1993; Robson *et al*., 1995). Here we have generated the mAb (7E4) directed against the proline-rich region of bacterial recombinant PbTRAP (details in Materials and methods). Sporozoites from clone 87-11 were stained by mAb 19F7 (Figure 3), while this mAb did not recognize wild-type *P.berghei* sporozoites. mAb 7E4 did not react against 87-11 sporozoites, but stained wild-type *P.berghei* sporozoites. These findings demonstrated that the mAb employed could distinguish PfTRAP from PbTRAP. The epitope recognized by mAb 19F7 has been mapped to the proline-rich region of PfTRAP (Robson *et al*., 1995). This region is highly divergent in length and sequence between PbTRAP and PfTRAP, with only 28% identical residues, which are mainly proline and glutamate. In IF, both 19F7 and 7E4 showed the same staining pattern, inside the sporozoite anterior and posterior to the nucleus, in 87-11 and *P.berghei* wild-type sporozoites, respectively. The expression of the circumsporozoite (CS) protein was unaffected in the transgenic parasite; both wild type and 87-11 showed a dense and uniform staining of the sporozoite surface using mAb 8.1 directed against *P.berghei* CS protein (Suhrbier *et al*., 1990) (Figure 3).

The infectivity of *P.berghei* sporozoites expressing PfTRAP for the vertebrate host was analysed by feeding 40 infected mosquitoes on two naive C57BL/6 mice. Blood-stage parasites were observed in Giemsa-stained blood films in both mice 5 days after exposure to the infective bites. PCR analysis on DNA extracted from these parasites revealed the presence of *PfTRAP* and the absence of *PbTRAP* (data not shown). These findings indicated that *P.berghei* parasites in which the endogenous *TRAP* gene has been replaced by PfTRAP are capable of completing the sporogonic cycle. To compare the infectivity of *P.berghei* wild-type and PfTRAP-expressing sporozoites, we have injected several groups of naive C57BL/6 mice with increasing numbers of wild-type and transgenic salivary gland sporozoites. This experiment revealed that the infectivity of both wild-type and 87-11 sporozoites was comparable. A minimum dose of 100 sporozoites from wild-type *P. berghei* and 87-11 parasites was required to initiate an infection in mice (Table I). The period between sporozoite injection and the appearance of first blood-stage parasites (prepatent period) was similar in wild-type and 87-11 parasites. C57BL/6 mice have been chosen for this study because of their high susceptibility to *P.berghei* infection. We obtained similar results when using the less susceptible BALB/c mice strain. In this case a dose of 1000 salivary gland sporozoites was needed to infect mice with a prepatent period of 5–6 days (data not shown). These results demonstrated that PfTRAP could replace PbTRAP in *P.berghei* and provided the rationale for using this clone as a reference for evaluating the phenotype of transgenic parasites expressing a mutated PfTRAP.

### *Development of transgenic parasites carrying a mutated PfTRAP gene*

*Anopheles stephensi* mosquitoes were fed on mice infected with the A-domain mutant clone L5-13 or the TRM mutant clone L8-9. Their development in the mosquito was monitored and compared with 87-11 and the TRAP knock-out L3-19. Sporozoite-containing oocysts of transgenic clones were easily observed in dissected midguts at day 14 p.i. No significant differences in the average number and size of the oocysts were observed (Table II). Sporozoites were collected from the midgut of infected mosquitoes dissected from day 16 to 18 p.i. To overcome the bias due to different infection levels in distinct mosquito batches, the number of recovered sporozoites in the gut was expressed as the mean number of sporozoites per oocyst. Again, no major differences could be detected by comparing the four transgenic clones; only clone L8-9 appeared to develop fewer sporozoites in the gut (Table II). The number of sporozoites in the mosquito salivary glands was assessed from day 21 to 24 p.i. While an average of 3750 sporozoites were detected in glands infected with reference clone 87-11, expressing the ITO 4 variant of PfTRAP, either none or very few sporozoites were recovered from salivary glands of mosquitoes infected with the A-domain mutant, the TRM mutant and the TRAP knock-out parasites (Table II). The calculated number of salivary gland sporozoites per oocyst indicated a 30- to

**Table I.** Infectivity of salivary gland sporozoites to C57BL/6 mice

| Clone[a] | No. of injected sporozoites | No. of infected mice[b] | Prepatent period[c] |
|---|---|---|---|
| Wild type | 20 | 0/3 | – |
| | 100 | 2/3 | 5.0 |
| | 1000 | 3/3 | 3.6 |
| | 10 000 | 3/3 | 3.0 |
| 87-11 | 20 | 0/3 | – |
| (replacement) | 100 | 7/11 | 5.5 |
| | 1000 | 13/13 | 5.0 |
| | 10 000 | 4/4 | 3.5 |

[a]*Plasmodium berghei* wild type and PfTRAP replacement clone 87-11.
[b]Number of mice infected out of the total number of mice injected.
[c]Period in days between injection of sporozoites and appearance of first blood-stage parasite.

**Table II.** Development of transgenic parasites in *A.stephensi*

| Clone[a] | Percentage of infected guts | Oocysts per gut | Sporozoites per gut | Sporozoites per salivary gland | Midgut sporozoites per oocyst | Salivary gland sporozoites per oocyst |
|---|---|---|---|---|---|---|
| 87-11 (replacement) | 75 ± 10 | 34 ± 13 | 18 000 ± 8485 | 3750 ± 1848 | 585 ± 260 | 150 ± 148 |
| L3-19 (knock-out) | 75 ± 8 | 41 ± 13 | 23 333 ± 13 769 | 18 ± 17 | 539 ± 189 | 0 |
| L5-13 (A-domain) | 86 ± 6 | 39 ± 12 | 20 500 ± 5635 | 170 ± 207 | 611 ± 407 | 5 ± 5 |
| L8-9 (TRM) | 83 ± 6 | 29 ± 14 | 8717 ± 2270 | 57 ± 38 | 333 ± 127 | 3 ± 2 |

All values are given with their standard deviation and are the mean of three independent infections. A minimum of 45 mosquitoes were analysed for the number of oocysts per gut and a minimum of 75 mosquitoes were dissected to determine the number of sporozoites per midgut and salivary gland for each parasite clone.
[a]PfTRAP replacement clone 87-11, TRAP knock-out clone L3-19, PfTRAP A-domain mutant clone L5-13, PfTRAP TRM deletion clone L8-9.

50-fold reduction of salivary gland invasion by both clone L5-13 and L8-9 compared with 87-11, while all transgenic parasite clones produced a similar number of midgut sporozoites per oocyst (Table II). These findings indicated that sporozoites carrying a mutation in either the A-domain or the TRM of PfTRAP behaved similarly to TRAP knock-out parasites (Sultan *et al*., 1997b) in their development in the mosquito vector; these sporozoites are strongly impaired in their ability to invade salivary glands.

No useful numbers of salivary gland sporozoites could be obtained from mosquitoes infected with the A-domain mutant, the TRM mutant or the TRAP knock-out clones. This limitation dictated the use of midgut sporozoites to compare the expression of PfTRAP by L5-13, L8-9, L3-19 and 87-11 parasites. As shown in Figure 4, anti-PbTRAP mAb 7E4 did not stain in IF sporozoites originating from the L5-13, L8-9, 87-11 and knock-out clones. At the same time, all sporozoites were stained by the anti-CS protein mAb 8.1. Expression of PfTRAP could be detected by using 19F7 in sporozoites from the clone 87-11, L5-13 and L8-9 sporozoites. As expected, the mAb 19F7 did not react with the TRAP knock-out parasites. Expression of TRAP is very weak in sporozoites obtained from midguts, as previously reported (Robson *et al*., 1995). However, the localization of the protein inside the parasite on both sides of the nucleus was identical for all TRAP-expressing sporozoites.

### *Gliding motility of the transgenic parasites*

We analysed the motility of the parasites by using the anti-CS protein mAb 8.1 in IF analysis to detect CS protein trails shed behind salivary gland sporozoites moving on glass slides (Stewart and Vanderberg, 1988). Circular and S-shaped trails were easily detected in wild-type sporozoites induced to glide at 37°C for 1 h (Figure 5). In most cases, the trails consisted of several circles of immunoreactive material. Gliding motility of wild-type *P.berghei* sporozoites varied between different preparations; in general >20% of the parasites left immunoreactive trails of CS protein after temperature induction. We also detected gliding motility in 87-11 sporozoites (Figure 5); however, most of the trails were short and did not exceed more than two turns. The percentage of motile 87-11 sporozoites was lower than that observed with wild-type sporozoites, ranging between 2 and 10%. To investigate this difference further, freshly dissected sporozoites from wild-type and transgenic 87-11 parasites were mixed at a ratio of 1:1 and exposed to 37°C. Sporozoite motility of each of the two clones in the mixed population was analysed by double IF using anti-CS protein mAb 8.1 in combination with either anti-PfTRAP 19F7 or anti-PbTRAP 7E4. As a control, sporozoites from the same preparations were induced to glide separately. Under these experimental conditions, wild-type and 87-11 sporozoites showed a similar gliding pattern. The percentage of motile parasites was similar and the trails were short. These findings would suggest that the apparent difference in motility when the clones were observed individually could be due to a non-specific effect of salivary gland debris in the different preparations.

For the two PfTRAP mutant clones L5-13 and L8-9, we performed motility assays with sporozoites collected from midguts at day 18 p.i. Sporozoites of this developmental stage are known to have a strongly reduced ability to glide when compared with salivary gland sporozoites (Vanderberg, 1974). The trails were considerably shorter, usually not exceeding 1–2 full circles. We could occasionally detect circular or S-shaped trails that could clearly be

identified as the result of gliding motility of 87-11 midgut sporozoites. The percentage of gliding sporozoites was below 1%. Faint and short trails of CS protein-reactive material were also shed by L5-13 sporozoites (PfTRAP A-domain mutant) upon exposure to 37°C (Figure 5). After analysing 3000 L8-9 midgut sporozoites from a series of independent mosquito infections, we failed to detect CS protein trails resulting from gliding motility.

### *Effect of PfTRAP mutations on the infectivity for the vertebrate host*

We have investigated whether the mutations introduced in the A-domain and TRM of PfTRAP affected sporozoite infectivity for the mammalian host. These experiments were performed by injecting mice with midgut sporozoites collected, at days 16–18 p.i., from the transgenic clones L3-19, L5-13, L8-9 and 87-11, as well as from *P.berghei* wild-type parasites. An increasing number of parasites were injected i.v. in naive C57BL/6 mice. The results shown in Table III originated from a series of independent experiments. Blood-stage infections were initiated by a minimum of 10 000 *P.berghei* midgut sporozoites. This finding is in agreement with previous observations showing that midgut sporozoites are significantly less infective than salivary gland sporozoites (Vanderberg, 1975; Touray *et al*., 1992). However, even injecting 10-fold more sporozoites of the same or different preparations did not always lead to 100% infection. By injecting 10 000 sporozoites of clone 87-11 we did not observe infections, whereas some mice developed a parasitaemia after the injection of 100 000 sporozoites. In this experiment the percentage of infected mice (31%) was similar to those observed using wild-type *P.berghei* midgut sporozoites (50%). As previously described, TRAP knock-out midgut sporozoites were not able to initiate the infection in the vertebrate host (Sultan *et al*., 1997b). Similarly, in our experiments up to one million TRAP knock-out sporozoites failed to infect C57BL/6 mice. Notably, 100 000 midgut sporozoites from clones L5-13 and L8-9 did infect one out of eight (12%) and two out of nine (22%) of the injected mice, respectively. These values are not far from the percentage (31%) of mice infected by injecting 100 000 PfTRAP wild-type midgut sporozoites. The prepatent period of all PfTRAP-expressing parasites was somewhat longer than that of wild-type *P.berghei*. The identity of the parasites recovered from the blood of the parasitized

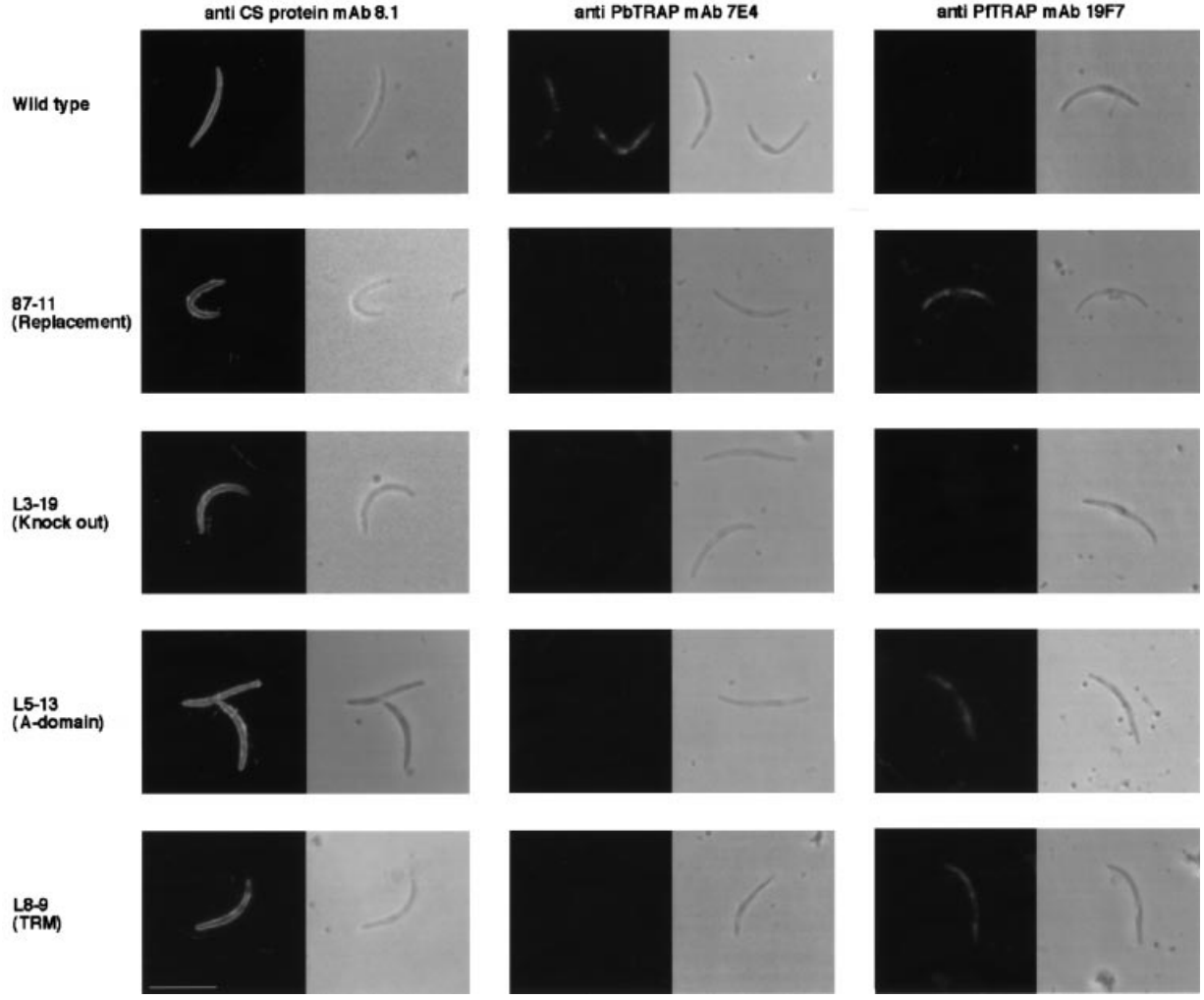



**Fig. 4.** TRAP expression in midgut sporozoites by transgenic parasites. Confocal fluorescence (dark field) and transmission microphotographs (bright field) of midgut sporozoites of *P.berghei* wild type, PfTRAP replacement 87-11, TRAP knock-out L3-19, PfTRAP A-domain mutant L5-13 and PfTRAP TRM mutant L8-9 (from top to bottom). The preparations were processed for IF using the mAbs 8.1, 7E4 and 19F7. Magnification ×630, plus zoom factor 2.0 for image acquisition. Scale bar = 10 μm.

mice was verified by PCR and restriction analysis (data not shown). These analyses ruled out contaminations or genetic reorganization events in the TRAP locus.

## Discussion

The development of gene-targeting technology in *P.berghei* has allowed a preliminary functional characterization of genes expressed during the mosquito stages (Ménard *et al*., 1997; Sultan *et al*., 1997b). Structure–function analysis of insertions, deletions or point mutations, in which the endogenous target gene is first disrupted and subsequently complemented by a mutant version, has been hampered by the availability of only one selectable marker. Two mechanisms of integration of homologous DNA are possible in *P.berghei* (Ménard and Janse, 1997). Single crossover of homologous DNA can lead to mutagenesis by insertion of a linearized plasmid (integration of a mutated copy into the target locus and concurrent disruption of the duplicated wild-type gene). However, this event has been shown to be reversible, leading to restoration of the wild-type gene (Ménard and Janse, 1997; Sultan *et al*., 1997b). Moreover, recent observations indicate that *P.berghei* possesses repair mechanisms to correct small mutations in homologous regions during the initial recombination event (Nunes *et al*., 1999). Gene replacement by double homologous recombination proved to overcome all these limitations. Our constructs were able to replace the endogenous *PbTRAP* gene with *TRAP* from a different *Plasmodium* species and to introduce the resistance marker without changing the expression pattern of the targeted gene. All DNA constructs contained three regions of homology with the genomic *TRAP* locus: two short regions at the end of the linearized construct and a longer central region downstream of the *TRAP* gene. We obtained the desired recombination event at the ends of the DNA construct in all successful transformations in spite of the presence of a central sequence of homology. This is consistent with the observation that the ends of a linear DNA molecule contribute more efficiently to the recombination event than does its central region (Hinnen *et al*., 1978). Previous reports have shown that transgenic *P.berghei* expressed correctly folded and processed AMA-1 of *P.falciparum* that was localized to the rhoptries of mature schizonts, similar to what is observed in *P.falciparum* (Kocken *et al*., 1998). Similarly, the expression and the localization of PfTRAP in transgenic sporozoites were indistinguishable from those of PbTRAP in wild-type *P.berghei* parasites. Our findings provide the first example of functional complementation by a heterologous gene in *Plasmodium*. Transgenic *P.berghei* parasites in which the endogenous *TRAP* gene has been replaced by *PfTRAP* of ITO 4 isolate efficiently completed the full malaria life cycle. We could not detect any difference in the infectivity of salivary gland sporozoites when comparing *P.berghei* wild-type to PfTRAP replacement sporozoites in the two mouse strains used. On the basis of these findings we concluded that TRAP does not restrict the host range infectivity of malaria sporozoites.

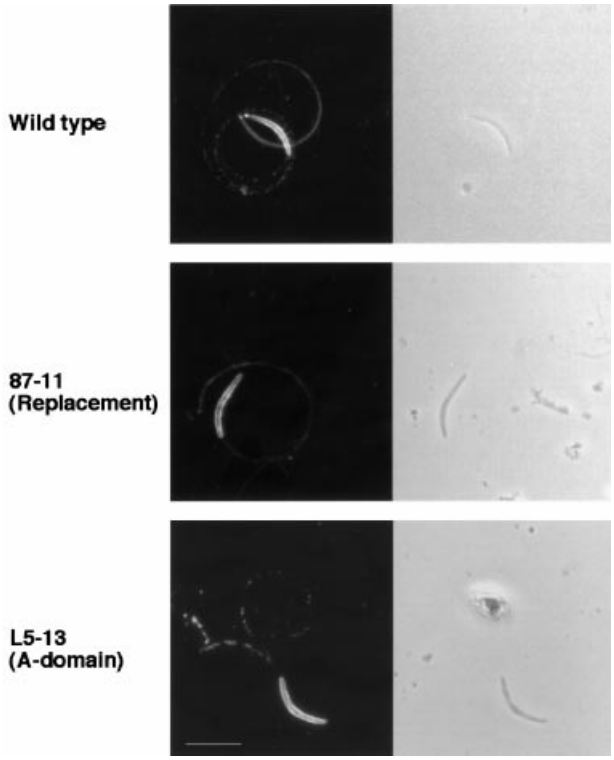



**Fig. 5.** Gliding motility of transgenic parasites. Salivary gland sporozoites of *P.berghei* wild type (top) and PfTRAP replacement 87-11 (middle) and midgut sporozoites of PfTRAP A-domain mutant L5-13 (bottom) were incubated at 37°C in culture medium before processing for confocal IF using anti-CS protein mAb 8.1. Motile sporozoites shed circular trails of material recognized by the CS protein antibody. The corresponding transmission microphotograph is shown on the right. Magnification ×630, plus zoom factor 2.0 for image acquisition. Scale bar = 10 μm.

**Table III.** Infectivity of midgut sporozoites to C57BL/6 mice

| Clone[a] | No. of injected sporozoites | No. of infected mice[b] | Prepatent period[c] |
|---|---|---|---|
| Wild type | 10 000 | 3/9 | 5.0 |
| | 100 000 | 4/8 | 4.5 |
| 87-11 | 10 000 | 0/6 | – |
| (replacement) | 100 000 | 4/13 | 6.0 |
| L3-19 | 100 000 | 0/2 | – |
| (knock-out) | 1 000 000 | 0/5 | – |
| L5-13 | 100 000 | 1/8 | 6.0 |
| (A-domain) | 400 000 | 2/8 | 5.5 |
| L8-9 (TRM) | 100 000 | 2/9 | 6.0 |

[a]*Plasmodium berghei* wild type, PfTRAP replacement clone 87-11, TRAP knock-out clone L3-19, PfTRAP A-domain mutant clone L5-13, PfTRAP TRM deletion clone L8-9.
[b]Number of mice infected out of the total number of mice injected.
[c]Period in days between infection and appearance of first blood-stage parasites.

TRAP is currently being investigated for its ability to elicit protective immunity in a sporozoite vaccine (Gilbert *et al*., 1997; Schneider *et al*., 1998) and in a multicomponent malaria vaccine (Tine *et al*., 1996; Ockenhouse *et al*., 1998; Shi *et al*., 1999). Previous reports have demonstrated that antibodies and cytotoxic T cells directed against TRAP of different *Plasmodium* species can interfere with sporozoite infectivity of hepatocytes (Potocnjak *et al*., 1980; Rogers *et al*., 1992; Müller *et al*., 1993). Our results demonstrate that the sporozoites can support considerable variations in the TRAP amino acid sequence without showing any apparent change in their infectivity. In fact, only 39% of the residues in PbTRAP and PfTRAP are identical. These findings may explain the sequence variability observed in PfTRAP from *P.falciparum* isolates (Robson *et al*., 1990, 1998) and highlight possible limitations of using this molecule as a vaccine.

PfTRAP and its homologues in *Plasmodium*, *Toxoplasma*, *Eimeria* and *Cryptosporidium* share an extracellular adhesive domain, a putative transmembrane sequence and a cytoplasmic tail (Naitza *et al*., 1998). This structural organization suggested that TRAP may function by connecting the parasite actin–myosin motor with external substrates or by delivering a specific signal upon binding to host ligands (Spaccapelo *et al*., 1997; Sultan *et al*., 1997b; Naitza *et al*., 1998; Sibley *et al*., 1998). The initial micronemal localization of TRAP, its adhesive properties and the impaired motility of TRAP knock-out sporozoites would support either of these hypotheses. Our results revealed that a single amino acid substitution in the A-domain or a deletion of the TRM severely impaired the ability of the sporozoites to invade salivary glands. Notably, sporozoites from the PfTRAP A-domain mutant parasites showed some degree of gliding motility as indicated by the presence of CS-protein-reactive trails. Altogether, these observations strongly indicated that TRAP is implicated in the recognition and invasion of salivary glands by the sporozoite, and that this process is functionally distinct from its involvement in parasite motility. The deletion of the entire TRM apparently impaired sporozoite motility. We could not detect a single parasite that had left a CS-protein-reactive trail upon induction at 37°C. The lack of motility of L8-9 sporozoites could explain their inability to invade salivary glands.

Midgut sporozoites from the two PfTRAP mutant clones L8-9 and L5-13 were able to infect naive mice almost as efficiently as 87-11 sporozoites carrying the wild-type *PfTRAP* gene. This observation demonstrated that although TRAP is crucial for sporozoite invasion of host hepatocytes, either the A-domain or the TRM can be structurally altered or eliminated without abolishing sporozoite ability to infect mice. The presence of an Arg-Gly-Asp (RGD) motif within the sequence of PfTRAP could explain the infectivity of L8-9 and L5-13 midgut sporozoites. PfTRAP is the only member of the protein family containing an RGD motif, which is conserved in all field isolates analysed (Robson *et al*., 1990). In other proteins this motif functions as a recognition sequence between extracellular glycoproteins and members of the integrin superfamily (Ruoslahti and Pierschbacher, 1987). Although there is no experimental evidence indicating a role for this motif in PfTRAP (Robson *et al*., 1995), we could not exclude the possibility that in *P.berghei* sporozoites the artificially introduced PfTRAP RGD motif could 'complement' for the lack of a disrupted adhesive domain in the TRM- and the A-domain PfTRAP mutants. Alternatively, the A-domain and the TRM could have a redundant function in the process of sporozoite invasion of liver cells recognizing similar ligands.

## Materials and methods

### *DNA constructs*

The screening of a *P.berghei* genomic library (Birago *et al*., 1996) with the *PbTRAP* gene as probe allowed the identification of two clones, pblibK and pblibA, that cover 0.8 kb of the *PbTRAP* upstream region and 2.8 kb of the *PbTRAP* downstream region, respectively. These clones were partially sequenced and used as templates in PCR. Oligonucleotides #106 (5′-GGGAAGCTTGTGATCCATGCGTGTAGTC-3′) and #107 (5′-GGGAAGCTTAATACGATTTCTTCTGGACAA-3′) amplified a 1 kb fragment 1.6 kb downstream of the *PbTRAP* stop codon. This PCR product was cloned as a *Hin*dIII fragment (restriction enzyme sites are underlined in the oligonucleotide sequences) in plasmid $pD_B.D_{TM}.D_B$ (Waters *et al*., 1997) at the 5′ end of the *DHFR-TS* transcription unit, which consists of the pyrimethamine-resistant form of the *T.gondii DHFR-TS* gene and the *P.berghei DHFR-TS* 5′ and 3′ UTRs. The *Hin*dIII site next to the *DHFR-TS* gene and a *Bam*HI site internal to the inserted DNA fragment were deleted by partial digestion, fill-in and religation, resulting in plasmid pTV. Oligonucleotides #1 (5′-GGGGGATCCATTTCTTTTTATAAGGG-3′) and #109 (5′-GGGGGTACCAAGCTTCCTAAATATGTTACAAC-3′) amplified a 0.8 kb fragment from the *PbTRAP* 5′ UTR immediately upstream of the *PbTRAP* ATG start codon. Oligonucleotides #2 (5′-GGGGGATCCTTTTAATAAACATATATATCTAG-3′) and #3 (5′-GGGGAGCTCGGTACCTGATGAATATGACCAAC-3′) amplified the 1.6 kb 3′ UTR of *PbTRAP* directly downstream of the TAA stop codon. Both products were cloned as *Kpn*I–*Bam*HI and *Bam*HI–*Sac*I fragments in pBluescriptSK, giving plasmid p53. The 146 bp N-terminal and 450 bp C-terminal regions of the coding sequence of *P.falciparum TRAP* were amplified from genomic DNA (ITO 4 isolate) (Robson *et al*., 1990) by oligonucleotides #99 (5′-GGGAGATCTATGAATCATCTTGGGAATGTTA-3′) and #100 (5′-GGGGGATCCACCTCATCATTACATACTTC-3′), and #101 (5′-GGGGGATCCATTCCATATTCACCATTACCTCC-3′) and #102 (5′-GGAGATCTTTAATTCCACTCGTTTTCTTCAGGTAA-3′), respectively. The PCR products were inserted as *Bam*HI–*Bgl*II fragments consecutively in the *Bam*HI site between the 5′ and 3′ UTRs in p53 (*Bam*HI and *Bgl*II have compatible overhangs and ligation leads to the loss of both sites). The insert was then cloned as a *Kpn*I fragment into plasmid pTV, giving pTV-1, which was used to generate the *TRAP* knock-out. The ITO 4, A-domain and TRM mutant *PfTRAP* constructs were generated by inserting the *PfTRAP* central 1090 bp *Bgl*II fragment into the *Bam*HI site of plasmid pTV-1. These *Bgl*II fragments were isolated from three *PfTRAP* expression plasmids used for *in vitro* TRAP-binding studies (F.Ghouze and A.Crisanti, unpublished). Insertion of wild-type *PfTRAP* sequences led to plasmid pTV-WT, the replacement construct. For plasmid pTV-A, an A to C point mutation at position 485 led to an Asp162 to Ala substitution and the creation of a *Pvu*II site ($CAGC_{485}TG$). In plasmid pTV-TRM, a 45 bp deletion at positions 748–792 resulted in an in-frame deletion of the 15-amino acid TRM motif and the loss of a *Kpn*I site. The *PfTRAP* transformation constructs were controlled by sequence analysis before their utilization.

### *Parasite transformation*

The *P.berghei* ANKA strain (clone 2.34) was used to generate transgenic parasites. Fifty micrograms of plasmid DNA were digested with *Hin*dIII (separates the insert from vector sequences) and *Bgl*I (cuts twice in the plasmid backbone), and purified by phenol–chloroform extraction. Electrotransformation of $10^8$ purified *P.berghei* schizonts with the linear DNA was performed at 1.1 kV and 25 μF in a Bio-Rad electroporator and the preparation injected i.v. in phenylhydrazine pre-treated Wistar rats as described previously (van Dijk *et al*., 1995; Waters *et al*., 1997). Pyrimethamine-resistant parasites were selected in the recipient rats and BALB/c mice as described (Waters *et al*., 1997), and cloned in BALB/c mice by limiting dilution.

### *Parasite DNA isolation and Southern blot analysis*

*Plasmodium berghei* DNA was prepared from blood-stage parasites. White blood cells were removed by filtration over Plasmodipur filters (Euro-

Diagnostics) and red blood cells lysed by incubation for 20 min on ice in 0.17 M ammonium chloride. Parasites were collected by centrifugation at 800 *g* for 10 min and lysed in 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA pH 8 with 1% SDS. After RNase A and proteinase K digestions (100 mg each) for 10 and 45 min, respectively, the DNA was purified by phenol–chloroform extraction and ethanol precipitated. The dried DNA was resuspended in 10 mM Tris–HCl, 1 mM EDTA pH 8 to concentrations of ~100 ng/μl. For Southern blot analysis ~2 μg of total DNA were digested with *Hinc*II, separated on a 1% agarose gel and blotted onto nylon Hybond-N+ membrane (Amersham). The following DNA fragments were used as probes: (a) an *Eco*RI fragment from plasmid pTV-WT encompassing 0.8 kb of *PbTRAP* 5′ UTR and 0.5 kb of *PfTRAP* sequence; (b) the *Kpn*I–*Sac*I insert from plasmid pblibA which encompasses 2.8 kb of the *PbTRAP* 3′ UTR and 1.6 kb of the *PbTRAP* gene; and (c) the *T.gondii DHFR-TS* gene amplified with oligonucleotides #123 (5′-AGAGGGGCATCGGCA-TCAAC-3′) and #138 (5′-GTCCGTGCGGGACGTAGCC-3′) from plasmid pTV-WT. All probes were gel-purified and 50 ng labelled using [α-$^{32}$P]dATP and the High Prime labelling kit (Roche). Hybridizations were performed overnight at 65°C. High stringency washes were performed twice for 15 min in 0.5× SSC, 0.1% SDS at 55°C for probes a and b (on account of the very high AT content of *Plasmodium* DNA), and at 65°C for probe c.

### *Analysis of parasite development and infectivity*

*Anopheles stephensi* mosquitoes (strain sd 500) were fed on parasitized BALB/c mice and maintained at 20°C in a humidified incubator. Mosquito batches fed on infected mice were analysed at day 14 p.i. when guts were dissected in phosphate-buffered saline (PBS) and oocysts detected by light microscopy. All preparations of sporozoites were carried out in RPMI 1640 (Gibco-BRL) without fetal calf serum. Mosquito guts were dissected at days 16–18 p.i. and homogenized, and sporozoites counted in a haemocytometer to determine the number of midgut sporozoites. Salivary glands were dissected at days 21–24 p.i. and homogenized, and the sporozoites counted. Midgut or salivary gland sporozoites were resuspended in RPMI and injected i.v. in the tail vein of naive C57BL/6 or BALB/c mice (minimum age of 6 weeks). Thin smears of tail blood were prepared daily up to day 10 p.i. and parasites stained with Giemsa. To determine the prepatent period, a minimum of 10 000 erythrocytes were analysed periodically on Giemsa-stained blood smears. For the analysis of sporozoite transmission by mosquito bites, C57BL/6 mice were anaesthetized, placed on top of a mosquito cage at day 21 p.i. and moved several times during the feeding to stimulate multiple probing by the mosquitoes.

### *Generation of PbTRAP monoclonal antibodies*

The $His_6$-tagged recombinant protein PbTRAP 2.1 was expressed in *Escherichia coli* and purified by nickel–chelate chromatography, as previously described (Müller *et al*., 1993; Robson *et al*., 1997). PbTRAP 2.1 recombinant protein encompassed the proline-rich region of PbTRAP (amino acids 263–429) (Robson *et al*., 1997). BALB/c mice were immunized intraperitoneally six times with 50 μg of purified PbTRAP 2.1. The last boost was performed with PbTRAP 2.1 coupled with *E.coli* β-galactosidase (Sigma) by glutaraldehyde cross-linking to enhance the immune response of the animals. Five days later the spleen of the immunized mice was removed and fused to X63 Ag 8.653 myeloma cells to generate mAb-producing hybrid cells. The culture supernatants from the growing hybrids were screened in ELISA and immunoblot against PbTRAP 2.1 and as a negative control against an unrelated $His_6$-tagged protein. The hybrid 7E4 was selected and cloned by limiting dilution. The culture supernatant of clone 7E4 ($IgG_{2a}$) was used in IF.

### *Immunofluorescence analysis*

Preparations of freshly dissected sporozoites resuspended in RPMI were spotted on microscope multiwell slides and incubated in humid chambers for 10 min at room temperature to allow sporozoites to settle. Sporozoites were further incubated for 1 h at 37°C to induce gliding motility. Prior to their application on slides, midgut sporozoite preparations were filtered over a 5 μm Nuclepore polycarbonate filter (Costar) to remove large debris. After incubation, excess medium was carefully removed, and slides were air-dried and kept at –20°C. For IF analysis the parasite samples were fixed in either 1% formaldehyde in PBS or in ice-cold acetone for 5 min. All further steps were performed at room temperature. Non-specific binding was prevented by incubating the slides in PBS containing 1% bovine serum albumin for 30 min. Sporozoites were then incubated for 1 h with either mAb 7E4 or 19F7 in the presence of 0.05% Triton X-100 or in the case of mAb 8.1 without Triton X-100. Bound antibodies were revealed by incubating the slides for 30 min with 1:15 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Becton Dickinson). For double-IF analysis, sporozoites were incubated as described above with either 19F7 or 7E4 followed by FITC-conjugated anti-mouse Ig. The parasites were then further incubated with biotinylated mAb 8.1 (1:50 000) for 1 h and bound CS protein antibodies revealed with rhodamine (TRITC)-conjugated streptavidin (1:1000) (Jackson ImmunoResearch Laboratories). IF analysis was carried out by using a Bio-Rad 600 confocal microscope.

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