Function of Region I and II Adhesive Motifs of *Plasmodium falciparum* Circumsporozoite Protein in Sporozoite Motility and Infectivity*

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The circumsporozoite protein of *Plasmodium falciparum* contains two conserved motifs (regions I and II) that have been proposed to interact with mosquito and vertebrate host molecules in the process of sporozoite invasion of salivary glands and hepatocytes, respectively. To study the function of this protein, we have replaced the endogenous circumsporozoite protein gene of *Plasmodium berghei* with that of *P. falciparum* and with versions lacking either region I or region II. We show here that *P. falciparum* circumsporozoite protein functions in rodent parasite and that *P. berghei* sporozoites carrying the *P. falciparum* CS gene develop normally, are motile, invade mosquito salivary glands, and infect the vertebrate host. Region I-deficient sporozoites showed no impairment of motility or infectivity in either vector or vertebrate host. Disruption of region II abolished sporozoite motility and dramatically impaired their ability to invade mosquito salivary glands and infect the vertebrate host. These data shed new light on the role of the CS protein in sporozoite motility and infectivity.

The malaria sporozoite has the unique ability to selectively invade the salivary glands of the mosquito vector and the vertebrate host cells (1). At this developmental stage the surface of the parasite is mainly covered by the circumsporozoite (CS) protein (2–4). In all malaria species the CS protein shows a similar structural organization consisting of a variable central region of repeats and two highly conserved motifs, regions I and II, placed at the amino- and carboxyl-terminal ends of the molecule, respectively. Region I is based around the short amino acid motif KLKQP. This motif is identical in all malaria parasites so far described in mammals (5). In the avian parasite *Plasmodium gallinaceum* the CS protein lacks region I. Region II is a 20-amino acid motif EWSXCXVTCGXG(V/I)XXRX(K/R) that shares sequence homologies to the type 1 repeat of human thrombospondin (TSP) (6). One to seven copies of this motif have now been found in a number of proteins involved in the complement pathway (properdin, C6, C7, C8A, C8B, and C9) as well as adhesive extracellular matrix proteins like ADAMTS, minidin, F-spondin, or SCO-spondin and micronemal proteins of apicomplexan parasites (7–13). One of these micronemal proteins, the thrombospondin-related adhesive protein (TRAP), contains a single TSP type 1 repeat and is expressed at the sporozoite stage of malaria parasites (8, 14).

Several proteins containing a TSP type 1 motif, including TRAP have the ability to selectively recognize glycosaminoglycans (GAGs) (12, 13, 15, 16). Similarly, recombinant forms of CS protein, as well as synthetic peptides encompassing region II, specifically bind to highly sulfated GAGs such as heparin and heparan sulfate (11, 17–21). It is intriguing that sporozoites express two distinct molecules, TRAP and CS protein, both containing a TSP type 1 repeat and having similar adhesive properties. The reasons for this are not clear. Gene disruption and biochemical evidence indicate that TRAP functions as a parasite receptor molecules and plays a crucial role in gliding, a form of substrate-dependent forward locomotion intimately linked to the process of sporozoite invasion of mosquito salivary glands and vertebrate host cells (22–24). The function of the CS protein is far less well established. The location on the parasite surface, the presence of the highly conserved region I and II sequences, and the adhesive property for GAGs have suggested that the CS protein ought to play an important role in the processes of sporozoite recognition and invasion of mosquito salivary glands and vertebrate host cells (17, 18, 25–27). Disruption of the CS gene did not add to the knowledge on the CS protein as receptor in the sporozoites. *Plasmodium berghei* parasites in which the CS gene has been targeted fail to develop into mature oocysts, and very few sporozoites if any are produced (28). The elucidation of the function of CS protein and its conserved motifs, regions I and II, would be of great importance for a better understanding of sporozoite interactions with mosquito salivary glands and vertebrate host cells. This information will also be useful to unravel functional relationships between TRAP and CS protein.

To examine the role of CS protein and its conserved motifs, we have generated transgenic *P. berghei* parasites in which the endogenous CS protein gene (*PbCS*) has been replaced with either the *P. falciparum* CS protein gene (*PfCS*) or with versions of *PfCS* without either region I or II. These parasites were studied throughout development in the mosquito as well as for their ability to infect mice.

**EXPERIMENTAL PROCEDURES**

*DNA Constructs—*The targeting constructs *pPCSP, pPCSP(RII−)*, and *pPCSP(RII+)* used in this study contained the following structural elements: (i) a 5′-untranslated region (UTR) of the *PbCS* gene encompassing nucleotides 1–1130 immediately upstream of the *PbCS* start
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Development of Transgenic Parasiotes—We have designed DNA constructs to achieve the deletion of the endogenous PbCS, the insertion of wild type (WT) or mutated PbCS versions, and the insertion of the selectable marker transcription unit, in a single transformation event. This strategy has several advantages. It generates non-reversible molecular events in contrast to gene targeting mediated by single crossover homologous recombination (22) and overcomes the parasite repair mechanism that has been shown in P. berghei to correct small mutations within the targeted gene with WT sequences (31). The replacement of the endogenous CS protein gene with a largely divergent homologous gene allows the structure-function analysis to be focused on the conserved motifs, thus facilitating the study of the transgenic parasites carrying a deletion of either region 1 or II. Moreover, the detection of the PbCS protein in P. berghei sporozoites offers the opportunity to analyze the expression of the replaced gene in single sporozoites. The construct pPfCSP contained the entire PbCS coding sequence (including the expressed CS protein) with the complete 5′UTR, and region I (24). For Southern blot analysis 5′UTR sequences (29) were introduced in the PbCS coding sequence by site-directed mutagenesis (Sculpl in vitro mutagenesis kit, Amerham Biociences) and verified by sequence analysis.

Parasite Transformation—The P. berghei ANKA strain (clone 2.34) was transformed in electroporation experiments by using 50–70 μg of a DNA insert. Plasmid DNA was digested to release the targeting inserts (~9.2 kb) from the plasmid backbone and purified by gel electrophoresis and phenol-chloroform extraction. Purified schizonts were transformed with the constructs pPfCSP, pPfCSP(RI) and pPfCSP(RII) using a Bio-Rad electroporator set at 1.1 kV and 25 microfarads and subsequently injected intravenously in phenylhydrazine (to increase reticuloocyte production)-treated Wistar rats as described previously (29). Pyrimethamine-resistant parasites were selected in the recipient rats and BALB/c mice as described previously (29) and cloned in BALB/c mice by limiting dilution.

Southern Blot Analysis—Genomic DNA was isolated from parasites as previously described (29). For Southern blot analysis 5 μg of genomic DNA was digested with EcoRV, separated on agarose gel, and blotted onto a nylon Hybond-N+ membrane (Amersham Biosciences). The following DNA fragments were used as probes: (a) a 666 bp fragment amplified from PbCS coding sequence with the primers CSFor81 (CCA GGA TAT GGA CAA GTG TTA TGC); (b) a 1.1-kb fragment amplified from PbCS with the primers PFCS3 (GGA CAA GGT CAC AAT ATG CCT); (c) a 520-bp fragment amplified from PfCS with the primers PFCS8 (GGA CAA GGT CAC AAT ATG CC) and PFCS9 (CAT ATA TAT TTC TAC AAT G), and (d) a 1.1-kb fragment amplified from the 3′-UTR sequence of PbCS amplified using the primers 5′-UTRICS (TTT AAA TAT GAT GTA GAT ATA G) and 5′-UTRCS2 (GGC TTT TAT TTC GTC CAG GTA TTA TGC); (b) a 666 bp fragment amplified from PbCS coding sequence with the primers CSFor81 (CCA GGA TAT GGA CAA AAT AAA) and CSRev53 (ATT GTT ATT ACC TCG TGC); (c) a 510 bp fragment amplified from PbCS with the primers CSFor81 (CCA GGA TAT GGA CAA GTG TTA TGC) and CSRev53 (CAT ATA TAT TTC GTC CAG GTA TTA TGC); (d) a 1.1-kb fragment amplified from the 3′-UTR sequence of PbCS gene with the primers 3′-UTRICS (ATA AAC ATG CAT GAT TA A) and 3′-UTRCS2 (GAG TAC TAC CGA ATC GAA AAG); and (e) a 520-bp fragment amplified from the DHFR-TS gene with the primers PDHFR1R (AGA GAA GGA GAA GCT GCA TCG TCA GCC AAG) and PDHFR1F (TTG AAA GAA GGA CAG TGC CCA CAG). All hybridization experiments were carried out as described (24). Parasite chromosomes were separated by pulse-field gel electrophoresis using a CHEF DRIII apparatus (Bio-Rad) at 600 s and 4 V/cm and run for 48 h. The gel was blotted and hybridized with probes b and c to detect PbCS and PbCS sequences.

Parasite Development in the Mosquito—Anopheles stephensi mosquitoes (strain sd 500) were fed for 1 h on WT and transgenic infected Balb/c mice having a parasitemia ranging from 6 to 7%. Mosquitoes were dissected at day 8, 14, and 21 post infection and processed to reveal the presence of oocysts and sporozoites in the guts and salivary glands. The dissection procedures and parasite counts were carried out in RPMI 1640 medium without serum.

Parasite Infectivity in Mice—Increasing numbers of WT and transgenic salivary gland and sporozoites were resuspended in RPMI and injected intravenously in the tail vein of naive C57BL/6 (minimum age 20 °C). Blood stage parasites from CSP-9, CSP(RI)-7 replicated normally in mice and generated gametocytes that developed into fertile gametes and morphologically normal ookinetes (data not shown). A. stephensi mosquitoes were fed on mice infected with these three different transgenic parasites. The development of the parasites in the mosquito was monitored starting from day 8 post infection, examining the oocyst where the expression of CS is first detected. Sporozoite-containing oocysts were detected in the gut at day 14 post infection (Fig. 2A). In four independent experiments CSP-9, CSP(RI)-6, and CSP(RII)-7 replicated normally in mice and generated gametocytes that developed into fertile gametes and morphologically normal ookinetes (data not shown). A. stephensi mosquitoes were fed on mice infected with these three different transgenic parasites.
similar to those observed for WT P. berghei parasites (Table I). This indicates that, unlike a CS knock-out where no sporozoites were detected in oocysts (28), parasite development in the gut was not affected by the replacement with PFCSP gene or with the variants without regions I or II. Compared with WT parasites, clones CSP-9 and CSP(RI–)–6 showed a 10- to 16-fold reduction in the number of salivary gland sporozoites, whereas a 290-fold reduction was observed with CSP(RII–)–7 parasites (Table I). P. falciparum and P. berghei WT sporozoites infected equally well the salivary glands of the mosquitoes utilized in this study (data not shown) thus arguing against the possibility that the reduced infectivity of the transgenic sporozoites is due to a salivary gland refractoriness of our laboratory mosquito that the reduced infectivity of the transgenic sporozoites is due to a salivary gland refractoriness of our laboratory mosquito strain for the selected PFCSP coding sequence. The infectivity of CSP-9 sporozoites was high enough to assess the effect of the deletion of regions I and II. Compared with CSP-9 and CSP(RI–)–6, CSP(RII–)–7 showed a greater than 95% reduction in the number of salivary gland sporozoites; statistical analysis indicated that such a difference is significant ($p < 0.05$). We further investigated the phenotype of the transgenic sporozoites by analyzing the expression of PFCSP protein by immunofluorescence (IF) and by assessing their ability to glide on glass surfaces, which reflects their motility status. This analysis demonstrated an initial normal location of the CS protein in all transgenic parasites. Freshly dissected gut sporozoites from each of the transgenic clones showed a uniform and bright surface staining when incubated with an antiserum against CS protein (data not shown). Following incubation at 37°C for 1 h, more than 50% of CSP-9 and CSP(RII–)–6 gut sporozoites (21 days post infection) showed CS protein-reactive trails from gliding on a glass surface (Fig. 2B). The shape of the trails, which consist of the immunoreactive CS material, indicated that about 10% of the sporozoites were able to form one or more complete loops, whereas the rest of the sporozoites left trails of variable shape and length. The frequencies of motile parasites were similar for WT, CSP-9, and CSP(RI–)–6 sporozoites and in agreement with previous reports on the motility of gut sporozoites (14). In contrast, CSP(RII–)–7 sporozoites did not show any form of gliding motility upon temperature switch to 37°C. At this temperature the uniform PFCSP protein surface staining observed at 25°C was replaced by clumps of immunoreactive material either outside the parasite body or in proximity to its surface (Fig. 2B). The inability of CSP(RII–)–7 sporozoites to glide was analyzed further by IF to study the expression and the location of TRAP, a micronemal parasite-encoded molecule that is implicated in sporozoite motility (22–24). TRAP expression was not altered in CSP(RII–)–7 sporozoites, a typical TRAP staining pattern with antibody-reactive material distributed within the cytoplasm both anterior and posterior to the nucleus (14) was observed in WT and in all transgenic sporozoites (Fig. 2B).

Infectivity of the Transgenic Parasites for the Vertebrate Host—The role of regions I and II for parasite infectivity of the mammalian host was investigated following intravenous injection into naive C57BL/6 mice of either salivary gland or gut sporozoites both collected at day 21 post infection. In addition, infected mosquitoes carrying the different transgenic parasites were allowed to feed on naive mice. A blood stage infection was initiated in a substantial fraction of the mice by as few as 100 WT salivary gland sporozoites (Table II), whereas with 1000 sporozoites all mice were infected. When 5000 sporozoites were inoculated, the number of days between injection of sporozoites...
and the first appearance of the parasites in the blood (the pre-patent period), decreased from 5.0 to 3.5 days. Identical rates of infection and pre-patent periods were observed by injecting similar numbers of CSP-9 and CSP(RI \textsuperscript{-6}) salivary gland sporozoites (Table II). CSP(RII \textsuperscript{-7}) sporozoites always failed to infect the mice (Table II). The infectivity of gut sporozoites was much less efficient than salivary gland sporozoites. This observation is in agreement with earlier reports showing that gut sporozoites are less infective than salivary gland sporozoites.

TABLE I

Parasite development in mosquitoes

| Parasite       | Oocysts per gut | Sporozoites per gut | Sporozoites per oocyst | Sporozoites per salivary gland | Salivary gland sporozoites per oocyst |
|----------------|-----------------|---------------------|------------------------|-------------------------------|--------------------------------------|
| WT             | 72 ± 47         | 20,318 ± 9,335      | 341 ± 127              | 17,166 ± 4,072                | 318 ± 197                            |
| CSP-9          | 78 ± 33         | 13,766 ± 5,919      | 210 ± 85               | 1,764 ± 1,235                 | 22 ± 12\textsuperscript{a}           |
| CSP(RI \textsuperscript{-6}) | 104 ± 36       | 19,138 ± 9,928      | 5,919                  | 1,088 ± 370                   | 11 ± 5\textsuperscript{a}            |
| CSP(RII \textsuperscript{-7}) | 67 ± 39         | 19,989 ± 12,883     | 345 ± 242              | 59 ± 7                        | 0.5 ± 0.7\textsuperscript{a,b}       |

\textsuperscript{a} Significant difference between WT and transgenic parasites.

\textsuperscript{b} Significant difference between CSP-9 and the transgenic parasite CSP(RII \textsuperscript{-7}).

TABLE II

Infectivity of salivary gland sporozoites

| Parasite       | Sporozoites injected | Mice infected | Pre-patent period\textsuperscript{a} |
|----------------|----------------------|---------------|---------------------------------------|
| WT             | 100                  | 2/3           | 5.0                                   |
|                | 1000                 | 7/7           | 3.8                                   |
|                | 3000                 | 2/2           | 3.5                                   |
|                | 5000                 | 4/4           | 3.5                                   |
| CSP-9          | 100                  | 3/7           | 5.0                                   |
|                | 1000                 | 9/9           | 3.8                                   |
|                | 3000                 | 2/2           | 3.5                                   |
|                | 5000                 | 4/4           | 3.5                                   |
| CSP(RI \textsuperscript{-6}) | 100                | 3/8           | 5.0                                   |
|                | 1000                 | 8/8           | 3.7                                   |
|                | 3000                 | 2/2           | 4.0                                   |
|                | 5000                 | 4/4           | 3.5                                   |
| CSP(RII \textsuperscript{-7}) | 100               | 0/4           |                                       |
|                | 1000                 | 0/3           |                                       |
|                | 5000                 | 0/2           |                                       |

\textsuperscript{a} Pre-patent period is the number of days between injection and first appearance of the parasites in the peripheral blood.

TABLE III

Infectivity of midgut sporozoites

| Parasite       | Sporozoites injected | Mice infected | Pre-patent period\textsuperscript{a} |
|----------------|----------------------|---------------|---------------------------------------|
| WT             | 500,000              | 1/2           | 8.0                                   |
|                | 300,000              | 2/4           | 8.0                                   |
|                | 100,000              | 6/11          | 9.5                                   |
|                | 10,000               | 0/14          | 0                                     |
| CSP-9          | 500,000              | 1/3           | 8.0                                   |
|                | 300,000              | 3/8           | 9.0                                   |
|                | 100,000              | 3/8           | 9.6                                   |
| CSP(RI \textsuperscript{-6}) | 500,000       | 1/2           | 8.5                                   |
|                | 300,000              | 2/6           | 8.0                                   |
|                | 100,000              | 4/11          | 9.6                                   |
| CSP(RII \textsuperscript{-7}) | 1,000,000            | 0/0           | 0                                     |
|                | 500,000              | 0/1           | 0                                     |
|                | 300,000              | 0/7           | 0                                     |
|                | 100,000              | 0/8           | 0                                     |

\textsuperscript{a} Pre-patent period is the number of days between injection and first appearance of the parasites in the peripheral blood.
sporozoites (32). Although a fraction of mice was infected with WT, CSP-9, and CSP(RI)-6 gut sporozoites, the region II-deleted parasites CSP(RII)-7 always failed to infect the mice even after the injection of 1 million sporozoites (Table III). Similarly, CSP(RII)-7-infected mosquitoes failed to initiate infection in the mice after blood feeding. These findings clearly indicate that, although the deletion of region I of CS protein does not impair sporozoite infectivity, region II has a critical role in this process.

**DISCUSSION**

Our data have important implications for understanding the function of the CS protein and its domains in the process of sporozoite motility and infectivity. All transgenic parasite lines developed normally to the oocyst stage. CSP-9 sporozoites infected mosquito salivary glands less efficiently than WT *P. berghei* sporozoites. Possibly the sequence changes introduced outside the conserved region I and II by replacing *PbCS* with *PfCS* could have reduced the ability of the *PfCS* protein to function optimally within the *P. berghei* background during sporozoite invasion of *A. stephensi* salivary glands. The removal of region I did not modify CSP(RI)-6 sporozoite motility and infectivity for mosquito salivary gland compared with CSP-9 parasites. This last finding would rule out a role of this motif in the process of sporozoite recognition of a mosquito salivary gland ligand. Such a conclusion is in agreement with the notion that the CS protein expressed by CSP(RI)-6 sporozoites has a structural organization similar to the *P. gallinaceum* CS protein (5). The absence of region I in *P. gallinaceum* CS protein does not affect parasite development into oocyst and sporozoite invasion of salivary glands.

The removal of region II almost abolished the ability of sporozoites to infect salivary glands. A role of region II in the process of CS protein recognition of salivary gland ligands could not be inferred on the basis of these findings. The deletion of this sequence also abolished sporozoite gliding thus making it difficult whether the impairment of salivary gland invasion was the direct consequence of disrupting a crucial receptor-ligand interaction or indirectly due to the lack of motility. CSP(RII)-7 sporozoites did not glide upon temperature switch to 37 °C as indicated by the lack of CS protein trails and the clumps of CS-reactive material along the surface and outside the parasite body. In these sporozoites the flow of CS protein from the apical complex to the caudal end of the parasite body is impaired. This phenotype sheds new light on the role in this process.

The phenotype of CSP(RII)-7 sporozoites sheds new light on the composition and the function of the sporozoite locomotion machinery. TRAP has been previously hypothesized to function in the process of sporozoite motility by linking host ligands bound to its adhesive domains (TSP type 1 repeat sequence and TSP type 2 repeat sequence) with the parasite actin-myosin motor (23, 24, 33). Notably, TRAP knock-out (22). This phenotype is identical to that of CSP(RII)-7 sporozoites thus indicating that TRAP alone is necessary but not sufficient for sporozoite motility. Our findings now demonstrate that for sporozoites to move TRAP must be functionally coupled to a CS protein containing an intact region II. This method could be achieved through a direct interaction or be mediated by additional parasite molecular partners. The deletion of region II in the CS protein could impair sporozoite motility either by disrupting these interactions or by impairing the ability of the CS protein to directly mediate attachment of the sporozoites to host ligands. The disruption of region II also abolished sporozoite infectivity for the vertebrate host as shown by the observation that CSP(RII)-7 sporozoites always infected the mice (Table II).

CSP-9 and CSP(RI)-6 sporozoites infected C57BL/6 mice equally well as the parental WT parasites thus demonstrating that the PICS protein is able to complement the activity of the endogenous *P. berghei* CS protein very efficiently. The disruption of region I did not impair sporozoite infectivity in the vertebrate host. This was a surprise, because region I is present in the CS protein of mammalian parasite and is absent in avian parasites. Sporozoites of mammalian parasites infect host hepatocytes, whereas avian parasites selectively invade and develop inside the macrophages. This correlation between CS protein structure and sporozoite infectivity for different host cell types has suggested a role of region I in sporozoite recognition and entry of vertebrate hepatocytes. If this were the case our findings indicate that this function can be complemented by region II at least in the parasite-vertebrate host combination utilized in this study. This would be in agreement with previous reports showing that the ligand of region I could be a GAG-related molecule sharing structural similarities with the ligand of region II (21, 34, 35).

The *P. falciparum* CS protein has been regarded as one of the best vaccine candidates for malaria (1, 36). Transgenic *P. berghei* sporozoites expressing *P. falciparum* CS protein represent a unique tool to systematically assess formulation, regimen, and molecular composition of a human malaria vaccine using a high throughput animal experimental model.

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