Role for the *Plasmodium* sporozoite-specific transmembrane protein S6 in parasite motility and efficient malaria transmission

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Summary

Malaria transmission occurs by intradermal deposition of *Plasmodium* sporozoites during the infectious bite of a female *Anopheles* mosquito. After formation in midgut-associated oocysts sporozoites actively enter mosquito salivary glands and subsequently invade host hepatocytes where they transform into clinically silent liver stages. To date, two sporozoite-specific transmembrane proteins have been identified that perform vital functions in natural malaria transmission. The sporozoite invasin TRAP drives sporozoite motility and target cell entry whereas the adhesin MAEBL mediates sporozoite recognition of and attachment to salivary glands. Here, we demonstrate that the sporozoite-specific transmembrane protein S6 is required for efficient malaria transmission to the vertebrate host. Targeted deletion of S6 results in severe impairment of sporozoite gliding motility and invasion of mosquito salivary glands. During sporozoite maturation S6 expression is tightly regulated by transcriptional and translational control. We propose that S6 functions together with TRAP/MIC2 family invasins to direct fast, efficient and specific cell entry and, ultimately, life cycle progression of the malaria sporozoite.

Introduction

Malaria remains the most important vector-borne infectious disease worldwide. It is caused by unicellular *Plasmodium* parasites that have the exceptional capacity to invade and develop within host erythrocytes. Malarial parasites are transmitted during the bloodmeal of an infected female *Anopheles* mosquito (Vanderberg and Frevert, 2004; Amino et al., 2006; Yamauchi et al., 2007). The contagious *Plasmodium* forms, sporozoites, are highly motile and actively enter the blood circulation in order to reach the liver where they undergo a dramatic transition and expansion phase. This pre-erythrocytic schizogony is clinically silent and results in the generation of thousands of pathogenic merozoites from a single sporozoite (Prudêncio et al., 2006). *Plasmodium* liver stage development compensates for the low numbers of transmitted sporozoites, a major bottleneck of the *Plasmodium* life cycle. Therefore, understanding the cellular and molecular mechanisms of sporozoite maturation, motility and invasion into host cells may assist in developing new potent intervention strategies against malaria.

*Plasmodium* sporozoites are formed inside oocysts, in a process termed sporogony, and share the unifying features of all apicomplexan invasive stages, i.e. they contain secretory organelles and display active locomotion (Sinden and Matuschewski, 2005). Sporozoites are covered with a dense coat made of circumsporozoite protein (CSP), the major surface coat protein (Nardin et al., 1982). Once mature, sporozoites become motile and egress from oocysts into the haemolymph (Aly and Matuschewski, 2005; Wang et al., 2005). Upon contact with their final target organ in the mosquito vector, the salivary glands, they specifically bind to and penetrate the distal portion of the lateral lobes resulting in accumulation of mature, infectious sporozoites in the salivary duct and potential transmission to the mammalian host.

The sporozoite-specific transmembrane surface protein TRAP (thrombospondin-related anonymous protein) is the founding member of a protein family that mediates cell invasion in Apicomplexan parasites (Tomley and Soldati, 2001). TRAP deficiency or mutations in key cytoplasmic and extracellular amino acid residues result in ablation of sporozoite locomotion and host cell entry (Sultan et al., 1997; Kappe et al., 1999; Wengelnik et al., 1999; Matuschewski et al., 2002a). The unifying structural features of TRAP/MIC2 family invasins are combinations of extracellular adhesive modules, i.e. the von Willebrand factor.
A-domain (A-domain) and the thrombospondin type I repeat (TSR), a cleavable transmembrane domain, and a cytoplasmic tail domain (CTD) that contains a penultimate tryptophan residue preceded by multiple negatively charged amino acids. According to the present model an extracellular binding event is transmitted to the cytoplasmic domain that links the transmembrane protein to the actin/myosin motor of the sporozoite (Keeley and Soldati, 2004; Schüler and Matuschewski, 2006). Up to date, TRAP remains the only known sporozoite-specific TRAP/MIC2 family protein that performs an essential role for locomotion and life cycle progression of the malaria sporozoite (Sultan et al., 1997; Matuschewski, 2006). Another sporozoite transmembrane protein, termed apical membrane antigen/erythrocyte binding-like protein (MAEBL), mediates salivary gland recognition and adhesion, but is dispensable for gliding locomotion (Kariu et al., 2002; Preiser et al., 2004; Fu et al., 2005; Sáenz et al., 2008).

In this study we characterized the in vivo function of a sporozoite-specific transmembrane protein, S6, which was initially identified in a screen for sporozoite-enriched transcripts (Kaiser et al., 2004). We show that S6 is important for efficient sporozoite locomotion and target cell entry. Apparently, *Plasmodium* sporozoites employ at least three stage-specific transmembrane proteins to guarantee efficient transmission to the mammalian host.

**Results**

**S6 is specifically expressed in Plasmodium sporozoites**

S6 (PF14_0404) was first discovered in a screen designed to identify *Plasmodium yoelii* sporozoite-specific genes that are absent in blood stages (Kaiser et al., 2004). The orthologous *Plasmodium berghei* S6 gene was identified in the genome database and encodes a protein of 2301 amino acid residues (Fig. 1A). The S6 protein appears to be a surface-exposed type I transmembrane protein and exhibits two main remarkable features. (i) It contains a carboxy-terminal TRAP family-like CTD, including the penultimate tryptophan and a cluster of negatively charged residues (Kaiser et al., 2004). These residues within the CTD are a hallmark of TRAP family invasins and play crucial roles during gliding locomotion (Kappe et al., 1999; Heiss et al., 2008). (ii) The large extracellular portion consists largely of low-complexity regions and lacks apparent cell adhesion modules, such as TSRs and A-domains.

We first analysed S6 transcript abundance during sporozoite maturation (Fig. 1B). cDNAs generated from ookinetes, oocyst, haemocoel and salivary gland-associated sporozoites were used as templates for semi-quantitative RT-PCR. The transcript of the major sporozoite surface protein CSP served for data standardization. *PbS6* expression was the highest in early mosquito stages, including ookinetes and young oocyst sporozoites. S6 was detectable in haemocoel sporozoites, but transcript levels were low in salivary gland-associated sporozoites. As observed previously (Matuschewski et al., 2002b), CSP transcription is slightly downregulated in mature salivary gland-associated sporozoites. In marked contrast, S6 can only be detected at higher cycle numbers, suggesting only residual transcript levels in mature sporozoites. In support of our findings, we independently isolated S6 as one of the most abundant transcript in a suppression subtractive hybridization screen to select for genes that are downregulated during sporozoite maturation (our unpublished data). Therefore, transcriptional control of S6 expression is markedly different from CSP and TRAP (Matuschewski et al., 2002b). Collectively, our data suggest that S6 transcription is downregulated during sporozoite maturation.

**S6 expression is translationally controlled**

We next wanted to examine protein expression during sporozoite formation and raised polyclonal antisera against *PbS6*. We first analysed protein expression during sporozoite maturation by Western blot analysis (Fig. 1C). Using the *PbS6* antiserum we detected a specific signal at the expected size of ~260 kDa. Unexpectedly, the S6 protein displays an increasing accumulation from oocyst to salivary gland sporozoites, differing substantially from the transcriptional profile, suggesting that S6 protein synthesis is delayed compared with gene transcription. This finding is reminiscent of translational repression, which has been previously observed for gametocyte-specific genes that are repressed by a DDX6 family member of DEAD-box RNA helicases, termed development of zygote inhibited (DOZI) (Mair et al., 2006).

To confirm the immunoblot analysis and detect the localization of *PbS6* we next studied wild-type (WT) sporozoites by immunofluorescence microscopy. In fixed haemocoel sporozoites we detected a punctuate pattern for *PbS6* that contrasted with the uniform distribution of CSP (Fig. 1D). In order to confirm the surface localization of S6 we treated sporozoites with the detergent Triton X-100 to remove the plasma membrane. Reactivity with either CSP or S6 antiserum was ablated in detergent-treated sporozoites suggesting a comparable localization. Together our findings indicate that the S6 protein localizes to the sporozoite plasma membrane and its expression is under stage-specific transcriptional and translational control.

**Generation of s6(−) parasites**

The tight expression regulation and spatial distribution of S6 is indicative of an important cellular function that is likely restricted to sporozoites in the mosquito vector. To identify the in vivo roles of S6 in *P. berghei* life cycle
progression we generated a Pbs6(−) parasite line by allelic exchange (Fig. 2A). Importantly, we could select for viable blood-stage parasites that contain a targeted deletion of the S6 open reading frame, in good agreement with sporozoite-specific gene expression and absence of transcripts in blood stages (Kaiser et al., 2004). The parental parasite populations were subcloned to generate clonal parasite lines, termed s6(−) (Fig. 2A).

Successful gene replacement in the s6(−) clones was confirmed by integration-specific PCR (Fig. 2B) and absence of S6 transcripts by RT-PCR analysis (Fig. 2C). Western blot analysis (Fig. 2D) confirmed successful S6 depletion by the replacement strategy and the specificity of the anti-S6 antiserum. In good agreement, s6(−) haemocoel sporozoites displayed no detectable immunofluorescence staining when incubated with the S6 anti-
serum (Fig. 2E). In conclusion, successful generation of S6 loss-of-function parasites demonstrated that this gene is dispensable for propagation of asexual blood-stage parasites.

Impairment of mosquito salivary gland invasion in s6(−) sporozoites

We next examined the fate of the s6(−) parasite lines during Plasmodium life cycle progression. Gametocyte formation, exflagellation, transmission to mosquitoes, as well as midgut infectivity (oocyst development and morphology) of s6(−) parasites were normal when compared with WT (data not shown). In general, oocyst development is complete at day 14 after mosquito infection. At this stage, s6(−) parasites were indistinguishable from WT parasites (Fig. 3A). Quantification of midgut-associated sporozoites revealed no differences between the two parasite lines. In marked contrast, s6(−) sporozoites were severely impaired in salivary gland invasion (Fig. 3B). The quantification of isolated salivary gland sporozoites showed a dramatic decrease in s6(−) parasite numbers when compared with WT. Importantly, the observed deficiency to enter mosquito salivary glands was accompanied by substantial accumulation of viable sporozoites in the mosquito haemocoel (Fig. 3C). These findings indicate that S6 is not involved in sporozoite invasion.
Deficient motility in s6(−) sporozoites

The observed deficiency in salivary gland invasion resembles the phenotype of trap(−) sporozoites, which lost their ability to invade target cells and perform active gliding locomotion (Sultan et al., 1997). We therefore compared gliding motility of mutant and WT sporozoites. Because s6(−) sporozoites are severely impaired in salivary gland invasion, we tested motility of sporozoites isolated from the mosquito haemocoel by indirect immunofluorescence staining of CSP deposited into the trails of gliding sporozoites (Fig. 4A). Only rarely did we detect sporozoites with trails, which displayed a more discontinuous pattern when compared with productive motility of WT sporozoites. A quantitative analysis of gliding locomotion revealed that on average 55% of adherent WT haemocoel sporozoites showed a trail of one circle or greater (Fig. 4B). In marked contrast, only very few sporozoites (3%) displayed trail patterns that were always discontinuous.

The vast majority of s6(−) sporozoites were active and displayed various patterns of non-productive motility, such as attached waving, bending and flexing, and pendulum-like movements (see also Movie S1). To confirm these findings we analysed sporozoite motility by phase-contrast microscopy (Fig. 4C). This analysis confirmed that sporozoite adhesion in vitro occurs normally in the absence of S6. S6(−) haemocoel sporozoites adhere with one end and display the immature and non-productive motility patterns, but apparently lost their ability to glide over long distances (Fig. 4C). Together, our findings demonstrate a crucial role for S6 in sporozoite locomotion.

S6(−) sporozoites are impaired in transmission to the mammalian host

We finally tested whether the observed defects in gliding locomotion translate into a complete block of transmission to the mammalian host. In order to quantify the invasion
capacity of s6(−) sporozoites we isolated haemocoel sporozoites by gentle perfusion of infected mosquitoes. When these sporozoites were added to subconfluent hepatoma cells and stained for mature liver stages at 48 h after sporozoite infection we consistently detected mature liver stages in s6(−)-infected hepatoma cells, albeit at greatly reduced numbers (Fig. 5A). Our quantitative analysis revealed a consistent reduction in liver stage numbers by at least an order of magnitude in vitro (Fig. 5B).

To exclude multiple roles of S6 during hepatocyte invasion and liver stage development, we incubated haemocoel sporozoites with subconfluent hepatoma cells and stained for mature liver stages at 48 h after sporozoite infection we consistently detected mature liver stages in s6(−)-infected hepatoma cells, albeit at greatly reduced numbers (Fig. 5A). Our quantitative analysis revealed a consistent reduction in liver stage numbers by at least an order of magnitude in vitro (Fig. 5B).

Table 1. Infectivity of s6(−) haemocoel sporozoites.

| Parasite population | Invasion | No. injected sporozoites | No. infected/No. injected | Prepatent period (days)
|---------------------|----------|--------------------------|---------------------------|----------------------|
| WT                  | 18%      | 1 000                    | 10/10                     | 4.7                  |
|                     |          | 10 000                   | 8/8                       | 4.6                  |
|                     |          | 100 000                  | 3/3                       | 3.3                  |
| s6(−)               | 0.5%     | 1 000                    | 1/10                      | 5.0                  |
|                     |          | 10 000                   | 8/8                       | 5.1                  |
|                     |          | 100 000                  | 5/5                       | 3.4                  |

a. Invasion was assessed by differential immunostaining of extra- and intracellular sporozoites.
b. Haemocoel sporozoites were injected intravenously at the numbers indicated.
c. Highly susceptible Sprague/Dawley rats were infected.
d. The prepatent period is the time until detection of the first blood stage by daily examination of Giemsa-stained blood smears.

blood-stage infections (Fig. 5C). This result confirms that a small proportion of s6(−) sporozoites retains the capacity to invade the salivary glands and is able to complete the entire Plasmodium life cycle. As expected, the observed defect in sporozoite locomotion and invasion to mosquito salivary glands translates into a substantial delay or absence of infection during natural transmission.

Table 1. Infectivity of s6(−) haemocoel sporozoites.
To confirm that the main role of S6 is during sporozoite entry of salivary glands, we bypassed the life cycle and injected haemoceol sporozoites intravenously into susceptible animals. Similar to WT parasites, s6(-) sporozoites induced patency in a dose-dependent manner in vivo (Table 1). This finding confirms that the observed defects in sporozoite locomotion and liver stage development in vitro are compensated for in vivo. Most importantly, reduced infectivity to the mammalian host is a consequence of the reduced capacity to invade the mosquito salivary glands.

**Discussion**

In this study we characterized a member of the parasite motor machinery that plays an important role for sporozoite gliding motility and entry into mosquito salivary glands. Depletion of S6 by a reverse genetics approach resulted in a consistent reduction of transmission of the malaria parasite to the mammalian host. We could show that the observed decrease in infectivity is a direct consequence of a specific reduction in the loads of mature sporozoites in the salivary glands. This defect in salivary gland invasion correlates with a striking phenotype in in vitro gliding motility. Thus, the most important cellular function of S6 is in mediating sporozoite accumulation in the final target organ of the mosquito vector through its role in sporozoite gliding locomotion.

*Plasmodium*, like other apicomplexan parasites, forms motile extracellular stages that actively penetrate biological barriers and enter target cells. These activities are driven by the parasite’s own motor machinery. Central components are transmembrane and surface proteins that link the outside world to the parasite’s actin/myosin motor. The founding member of the parasite family of invasins, TRAP, is vital for sporozoite motility and cell invasion (Sultan *et al.*, 1997) and appears to function throughout the parasite’s journey from the oocysts to the mammalian liver (Sultan *et al.*, 1997; Kappe *et al.*, 1999; Wengelnik *et al.*, 1999; Matuschewski *et al.*, 2002a). The parasite apparently expresses tailor-made TRAP/MIC2 family invasins for different life cycle stages. For instance,

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ookinetes express the invasin circumsporozoite/TRAP-related protein (CTRP) that is essential for ookinete motility and midgut penetration (Dessens et al., 1999; Templeton et al., 2000) and can be functionally grouped into the TRAP/MIC2 family (Heiss et al., 2008). Sporozoites are arguably the most versatile extracellular parasite stages that need to breach numerous barriers along their journey (Amino et al., 2006; Baer et al., 2008; reviewed in Frevert et al., 2008). Not surprisingly, sporozoites express additional TRAP/MIC2 family proteins that together permit efficient life cycle progression. One member, TRAP-like protein (TLP), contains two extracellular adhesion domains, the TSR and the A-domain, yet plays a redundant role in all aspects of sporozoite biology (Heiss et al., 2008; Moreira et al., 2008). In marked contrast, s6(–) mutants display pronounced defects in sporozoite gliding motility and salivary gland invasion. Therefore, S6 functions appear to closely resemble those of TRAP (Sultan et al., 1997).

However, two important features differ fundamentally between the two proteins. (i) While TRAP contains classical adhesion modules, namely an A-domain and a TSR, in its extracellular portion, the extracellular region of S6 appears to be lacking any typical adhesion modules. (ii) The observed defects in the s6(–) mutants are most prominent in the mosquito phase of the Plasmodium life cycle. trap(–) mutants, in contrast, fail to infect the mammalian host in vitro and in vivo. Most strikingly, the two proteins do not have redundant but rather distinct roles. Therefore, parasite motility and host cell entry are driven by an array of extracellular proteins that each mediate individual steps from initial substrate and cell recognition to target cell penetration. S6(–) parasites largely lost their capacity to glide productively in vitro and to accumulate inside salivary glands.

Our findings show that active parasite locomotion, as displayed by haemocoeal sporozoites, is necessary for salivary gland entry. In addition to the general sporozoite invasin TRAP (Sultan et al., 1997) the malaria parasite employs two stage-specific genes, MAEBL and S6, that function in salivary gland adhesion (Kariu et al., 2002) and sporozoite motility respectively. Most importantly, we identified by experimental genetics S6 as the second sporozoite-specific Plasmodium protein that has an important role in transducing the motor force from the parasite interior to the extracellular substrate.

**Experimental procedures**

**P. berghei life cycle**

Anopheles stephensi mosquitoes were raised at 28°C, 75% humidity, under a 12 h light/dark cycle, and maintained on a 10% sucrose solution during adult stages. For infections with clonal P. berghei parasites (ANKA strain, GFP-507cl; Janse et al., 2006), 4-day-old female mosquitoes were fed on anaesthetized NMRI mice, which had been infected with P. berghei WT parasites or the isogenic s6(–) parasites. Parasitaemia was determined for the presence of gametocyte-stage parasites capable of exflagellation. After infection the mosquitoes were maintained at 20°C and 80% humidity. Dissections were performed at days 10, 14–18, to determine infectivity, and perform a detailed spatial and temporal analysis of the sporozoite populations. Oocyst, haemocoel and salivary gland-associated sporozoites were separated and analysed as described (Vanderberg, 1975).

**S6 expression analysis**

Detection of S6 transcripts was performed by semi-quantitative RT-PCR. A total of 5 × 10⁶ WT sporozoites were collected from oocysts, haemocoeal or salivary glands, and poly (A) RNA was isolated using oligo dT-columns (Invitrogen). cDNA synthesis was performed after DNase I digestion (Ambion) with random decamer primers (Ambion). S6 transcript abundance was determined using primers S6Rev (5′-GGGTATACACCTTCACTATTATC-3′) and S6RTrev (5′-TCCTACCTTTGCCAAATAATACAG-3′) and CSP primers CSPfor (5′-GACGATTCTTATATCCAAGGCCG-3′) and CSPRev (5′-CCCTAATGAATGCTTCAATTAAATATCTTG-3′) for normalization.

**PbS6 gene targeting**

For targeted deletion of PbS6 the gene replacement approach was followed (Thathy and Ménard, 2002). Two targeting fragments were selected from the non-coding regions of S6 and amplified with P. berghei genomic DNA as a template using the following primers: (i) S6rep1for (5′-TCCCCGGCGGGCATTAAATATGCGCG-3′; SacII site is underlined) and S6rep1rev (5′-CGGGATCCCTTTACTCGGTTGTCTATGAATG-3′; BamHI site is underlined) for a 1045 bp fragment of the 5′ UTR; (ii) S6rep3for (5′-CCCCCCAGCCTTATATAGACATGGACACAAGAGGATTACGC-3′; HindIII site is underlined) and S6rep4rev (5′-GGGGTGACCTCTACAGAAATCCTACACTATGATGCC-3′; KpnI site is underlined) for a 807 bp fragment of the 3′ UTR. Cloning of the two fragments into the P. berghei targeting vector flanking the Tgdhfr/ts-positive selection marker that provides resistance to the antifolate pyrimethamine resulted in the plasmid pMS01. The targeting vector was linearized with KpnI/SacII and parasite transfection, positive selection and parasite cloning were performed with the fluorescent P. berghei ANKA strain as described (Janse et al., 2006). Three independent s6(–) clonal parasite populations were obtained and tested for Plasmodium life cycle progression. The detailed phenotypical analysis was performed with one representative clone. Replacement-specific PCR amplifications of the s6(–) locus was performed with specific primer pairs that amplify either the WT or the mutant gene loci.

**Western blotting and immunofluorescence**

To generate polyclonal antisera a recombinant amino-terminally His-tagged S6 polypeptide encompassing 156 central amino acid residues, selected for favourable antigenicity, was expressed in an Escherichia coli expression vector in BL21 (DE3) cells. The purified recombinant protein was used to raise polyclonal antibodies in pre-screened rabbits housed in a SPF facility.
To score sporozoite invasion a two-colour assay was performed with ice-cold methanol and re-labelling with the Alexa-Fluor 546-conjugated anti-rabbit and anti-mouse antibodies respectively (Sigma-Aldrich).

For sporozoite immunostaining and gliding motility assays haemocoeol sporozoites of WT and s6(–) parasites were isolated by gentle perfusion in RPMI/3% bovine serum albumin (BSA) at days 15–16 post feeding. Pooled haemocoeol sporozoites were transferred to BSA-precoated glass coverslips, incubated for 15 min at 37°C in a humid chamber, and fixed in 4% formaldehyde. For membrane extraction, parasites were treated with 1% Triton X-100 (Sigma) in PBS prior to fixation (Bergman et al., 2003). For permeabilization sporozoites were incubated with 0.05% Saponin (Sigma) in PBS/1% FCS. Non-permeabilized and permeabilized sporozoites were incubated with the S6 polyclonal antiserum (1:100) or the monoclonal anti-CSP antibody (1:10000). Bound antibodies were detected with Alexa Fluor 546-conjugated anti-rabbit IgG and Alexa-Fluor 488-conjugated anti-mouse IgG respectively (Molecular Probes).

Analysis of sporozoite infectivity

To determine sporozoite infectivity in vivo, s6(–) and WT haemocoeol sporozoites were injected intravenously at the numbers indicated into young Sprague/Dawley rats. Patency was checked daily by Giemsa-stained blood smears for at least 14 days. The prepatent period is defined as the time until occurrence of the first blood-stage parasites.

For determination of sporozoite infectivity in vitro, haemocoeol sporozoites were added to subconfluent monolayers of HuH7 cells at the numbers indicated, incubated for 90 min at 37°C and washed off. Liver stages were revealed after 48 h using primary antibodies against P. berghei HSP 70 (Tsui et al., 1994) and Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes). To score sporozoite invasion a two-colour assay was performed as described previously (Rénia et al., 1998). Briefly, HuH7 cells were grown to subconfluency, incubated for 90 min with the haemocoeol sporozoite suspension and washed in DMEM medium containing 10% FCS. To distinguish between extra- and intracellular parasites, extracellular parasites were labelled with the monoclonal anti-CSP antibody, followed by Alexa-Fluor 546-conjugated anti-mouse IgG (Molecular Probes). Permeabilization was performed with ice-cold methanol and re-labelling with the monoclonal anti-CSP antibody, followed by Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes).

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been submitted to the GenBank database with the Accession No. FJ160771.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

\textbf{Movie S1.} Non-productive gliding motility of \textit{s6(−)} haemocoel sporozoites on glass slides, co-incubated with 3% bovine serum albumine. Total time: 20 sec.

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