Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection

Ahmed S. I. Aly,1 Sebastian A. Mikolajczak,1 Hilda Silva Rivera,1 Nelly Camargo,1 Vanessa Jacobs-Lorena,1,2 Mehdi Labaied,1 Isabelle Coppens3 and Stefan H. I. Kappe1,2*
1Seattle Biomedical Research Institute, Seattle, WA 98109, USA.
2Department of Global Health, University of Washington, Seattle, WA 98195, USA.
3Department of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA.

Summary

Malaria parasite sporozoites prepare for transmission to a mammalian host by upregulation of UIS (Upregulated in Infectious Sporozoites) genes. A number of UIS gene products are essential for the establishment of the intrahepatocytic niche. However, the factors that regulate the expression of genes involved in gain of infectivity for the liver are unknown. Herein, we show that a conserved Plasmodium sporozoite low-complexity asparagine-rich protein, SAP1 (Sporozoite Asparagine-rich Protein 1), has an essential role in malaria parasite liver infection. Targeted deletion of SAP1 in the rodent malaria parasite Plasmodium yoelii generated mutant parasites that traverse and invade hepatocytes normally but cannot initiate liver-stage development in vitro and in vivo. Moreover, immunizations with Psap1(−) sporozoites confer long-lasting sterile protection against wild-type sporozoite infection. Strikingly, lack of SAP1 abolished expression of essential UIS genes including UIS3, UIS4 and P52 but not the constitutively expressed genes encoding, among others, sporozoite proteins CSP and TRAP. SAP1 localization to the cell interior but not the nucleus of sporozoites suggests its involvement in a post-transcriptional mechanism of gene expression control. These findings demonstrate that SAP1 is essential for liver infection possibly by functioning as a selective regulator controlling the expression of infectivity-associated parasite effector genes.

Introduction

The first step of malaria transmission is the injection of sporozoites into a mammalian host by an anopheline mosquito bite (Vanderberg and Frevert, 2004; Amino et al., 2006). Initially, sporozoites form in mosquito midgut oocysts and subsequently invade and reside inside the salivary glands (reviewed in Matuschewski, 2006). In the mosquito salivary glands, sporozoites gain infectivity that is critical to support their transmission and life cycle progression in the mammalian liver (Vanderberg, 1974; 1975). Previous work has demonstrated that gain of infectivity is accompanied by extensive differential upregulation of unique gene products called UIS (Upregulated in Infectious Sporozoites) (Matuschewski et al., 2002). Indeed, UIS genes were shown to be essential for malaria parasite liver infection. UIS3 and UIS4 (Mueller et al., 2005a,b; Tarun et al., 2007) are proteins of the parasitophorous vacuole membrane (PVM), the principal host–parasite interface during cell infection (Mueller et al., 2005b; Mikolajczak et al., 2007a). Deletion of UIS3 and UIS4 leads to complete early arrest of liver-stage development inside the PVM (Mueller et al., 2005a,b; Tarun et al., 2007). Recently, it was shown that UIS3 interacts with liver fatty acid-binding protein (L-FABP) indicating a potential role of this protein in fatty acid uptake from the host hepatocyte (Mikolajczak et al., 2007a). Simultaneous deletion of the UIS gene P52 (also called P36p), a putative GPI-anchored protein, and a non-UIS gene, P36, a putative secreted protein, renders sporozoites unable to form a PVM during infection and leads to complete developmental arrest at the early stage of hepatocyte infection (van Dijk et al., 2005; Ishino et al., 2005a; Labaied et al., 2007). Therefore, a number of UIS proteins critically contribute to establishing the intracellular parasitic niche either by the formation or modification of the host–
parasite interface (reviewed in Mikolajczak and Kappe, 2006). However, it remains unknown what factors regulate the expression of UIS genes and consequently liver infectivity of sporozoites. Herein, we have identified a cytoplasmic low-complexity asparagine-rich protein, SAP1 (Sporozoite Asparagine-rich Protein 1) that is essential for liver infection possibly by means of regulating the expression of effector proteins such as P52, UIS3 and UIS4. Targeted deletion of PySAP1 generated mutant parasites that traverse host cells, invade hepatocytes and form a PVM but cannot initiate liver-stage development and consequently completely lose mammalian infectivity in vivo. Drastically reduced transcript levels of liver infection-associated UIS genes in SAP1-deficient sporozoites in combination with SAP1’s putative cytoplasmic localization suggest a post-transcriptional regulation of gene expression function of SAP1 in malaria parasite liver infection.

Results

**SAP1 is a conserved Plasmodium sporozoite protein with an asparagine-rich low-complexity domain**

We searched for putative cytoplasmic proteins that are highly expressed in sporozoites but not in blood stages because they might uniquely contribute to regulation of sporozoite infectivity. SAP1 was first identified as a sporozoite-expressed gene in a suppression subtractive hybridization (SSH) screen of *Plasmodium yoelii* salivary gland sporozoites versus blood-stage merozoites (designated S22, sporozoite-specific gene 22) (Kaiser et al., 2004). *PySAP1* (gene identifier PY03269) has orthologues in other *Plasmodium* species including the human malaria parasite *P. falciparum* (gene identifier PF11_0480) (Carlton et al., 2002; Gardner et al., 2002). Alignment of *PISAP1* and *PySAP1* revealed that the *PySAP1* open reading frame (ORF) was incomplete. Bioinformatics analysis and direct sequencing revealed that the *PySAP1* coding sequence was dispersed over two unassembled sequence contigs (described in Text S1 in Supplementary material). We confirmed the overlap between the two contigs encoding *PySAP1* by genomic PCR and reverse transcription polymerase chain reaction (RT-PCR) analysis. The correct start and stop codons as well as the correct exon–intron organization were also confirmed by RT-PCR analysis of salivary gland sporozoite RNA (Fig. 1A). The corrected *PySAP1* ORF nucleotide sequence (9723 nucleotides) and the predicted protein sequence (3240 amino acids) were deposited in NCBI GenBank (Accession No.: EU652769, Text S1). *PySAP1* encodes a large putative protein with a predicted 370 kDa molecular mass. *PySAP1* has one large exon followed by two small exons (Fig. 1A). Signal sequences, transmembrane domain(s), enzymatic or structural motifs were not identifiable in any of the predicted *Plasmodium* SAP1 protein sequences examined. SAP1 proteins are characterized by the presence of an extended internal asparagine-rich low-complexity domain, with an asparagine content of 27% in *P. yoelii* flanked by predicted globular domains with low asparagine content (Fig. 1B). Interestingly, these (N)- and (C)-terminal regions are highly conserved among *Plasmodium* species. The *PySAP1* N-terminus shares 70% amino acid sequence identity with the N-terminus of the *P. falciparum* orthologue, and the *PySAP1* C-terminus shares 89% amino acid sequence identity with C-terminus of *PfSAP1* (Fig. 1B). However, the overall amino acid sequence identity of SAP1 between *P. yoelii* and *P. falciparum* is only 26% due to the sequence divergence in the asparagine-rich domain (Fig. 1B).

**Sporozoite-specific expression profile of PySAP1 and PISAP1**

RT-PCR analysis revealed that *PySAP1* is transcribed in oocyst and salivary gland sporozoites (Fig. 1C). As expected from the results of the previous SSH screen (Kaiser et al., 2004), no transcripts were detected in unsynchronized mixed blood stages (Fig. 1C). A similar expression pattern of *SAP1* was observed in *P. falciparum* oocyst and salivary gland sporozoites. No transcripts were detected in mixed blood stages. (Fig. 1D). Therefore, the sporozoite-specific expression profile of *PISAP1* is similar to the expression profile of *PySAP1*.

**Localization of PySAP1**

To determine the cellular localization of SAP1, we generated rabbit polyclonal antisera against a peptide in the C-terminus of *PySAP1* and tested the antisera in immunofluorescence assays (IFAs) using *P. yoelii* sporozoites. A specific sporozoite-internal staining that excluded the nucleus and was distinct from circumsporozoite (CS) protein staining was observed (Fig. 2). SAP1 localization appeared uneven and clustered within the sporozoite cytoplasm. Its localization appeared also distinct when compared with cytoplasmic heat shock protein (HSP70) staining, which was induced by incubation of the sporozoites at 37°C for 1 h (Fig. 2). SAP1 staining was only observed in sporozoites after membrane permeabilization, indicating that SAP1 localizes exclusively to the interior of the sporozoite. Together, the data suggest that SAP1 localizes to the cytoplasm, intracellular organelles or other structures within the cytoplasm as predicted by the lack of a secretory signals in the SAP1 sequence. Pre-immune sera did not show reactivity with sporozoites (data not shown).
Targeted deletion of PySAP1 and knockout phenotype in blood and mosquito stages

Targeted gene deletion of PySAP1 was conducted by double-cross-over homologous recombination to replace the majority of the coding sequence with the TgDHFR/TS selection marker cassette (Fig. 3A) (Menard and Janse, 1997). Deletion-specific genomic PCR analysis confirmed the successful double-cross-over recombination event and the successful isolation of a Pysap1(−) parasite clone with pure gene deletion background (Fig. 3B). Therefore, PySAP1 was successfully deleted in the erythrocytic stages with no observed deficiency of blood-stage development (data not shown). In addition, the morphology of male and female gametocytes in thin infected-blood smears and male gamete exflagellation in wet mounts of infected blood were indistinguishable from P. yoelii wild-type (WT) parasites (data not shown). Transmission of Pysap1(−) parasites to mosquitoes resulted in normal midgut infection and oocyst development. Pysap1(−) oocyst sporozoites developed in a similar manner as PyWT oocyst sporozoites (Table S1 in Supplementary material). Importantly, Pysap1(−) sporozoites accumulated in the salivary glands in numbers comparable to WT, indicating normal salivary gland infection (Table S1). RT-PCR analysis confirmed the absence of PySAP1 transcripts in Pysap1(−) sporozoites (Fig. 3C). IFAAs with the anti-SAP1 antisera were negative, confirming that Pysap1(−) sporozoites do not express SAP1 (Fig. 2). The data give further support to the specificity of the SAP1 antisera. We conducted all initial experiments with two independent clones of Pysap1(−) that were identical in their phenotypes (data not shown). Thereafter, experiments were conducted with a single Pysap1(−) clone.

Fig. 1. SAP1 gene structure, protein structure, conservation among Plasmodium species and transcriptional profiling. A. A schematic representation of the SAP1 gene organization: arrows show the locations of primers used for RT-PCR to identify the start and stop codons as well as exon 2 and exon 3. B. Alignment of the putative PySAP1 with PbSAP1 and PfSAP1. The asparagine-rich regions are shown as light grey boxes bordered by non-asparagine-rich N- and C- termini shown as dark grey boxes. Total amino acid sequence identities to PySAP1 are shown to the right and amino acid sequence identities between the N-terminal and the C-terminal are shown inside their respective boxes. Per cent (%) asparagine content is shown only for PySAP1. C. RT-PCR analysis of RNA isolated from P. yoelii sporozoites shows the expression of PySAP1 in ooSPZ (oocyst sporozoites) and sgSPZ (salivary gland sporozoites) but not in mixed blood stages (mixedBS). PyCS (circumsporozoite protein) is a positive RT-PCR control for sporozoite expression and PyHSP70 is a positive RT-PCR control for mixed blood stages. D. RT-PCR analysis of different P. falciparum life cycle stages shows expression of PfSAP1 in ooSPZ and sgSPZ but a lack of expression in blood stages (mixedBS).
Pysap1(-) sporozoites fail to induce blood-stage infection and elicit sterile protection against PyWT sporozoite challenge

We tested the infectivity of PySAP1-deficient salivary gland sporozoites in susceptible BALB/c mice. Mosquito bite experiments with more than 50 Pysap1(-)-infected mosquitoes/mouse did not result in blood-stage infection (data not shown). Strikingly, intravenous (iv) injection of escalating doses of Pysap1(-) salivary gland sporozoites did not lead to blood-stage parasitaemia, tested daily by blood smears until day 14 post infection (Table 1). Even with extremely high doses of more than 2 million sporozoites no subsequent blood-stage parasitaemia was observed. This highest dose corresponded to a ~200 000-fold increase over the minimal infectious dose of PyWT sporozoites administered to BALB/c mice by iv injection (Belmonte et al., 2003). Hence, we conclude that PySAP1 is essential for parasite pre-erythrocytic stage functions after transmission from the mosquito to the mammalian host.

We next tested whether Pysap1(-) salivary gland sporozoite immunization of mice can induce sterile protection against PyWT sporozoite challenge. Four groups of BALB/c mice were immunized iv with three doses of 10 000 Pysap1(-) salivary gland sporozoites, in 2-week intervals (Table 2). The first immunization group (group I) was challenged by iv injection of 10 000 PyWT sporozo-
tes at day 7 after the last immunization dose. Two of the immunization groups (groups II and III) were challenged by iv injection of 10,000 PyWT sporozoites 30 and 210 days after the last immunization dose. The mice of group III were then challenged by PyWT erythrocytic stages 2 weeks after the last challenge with either 10^3 or 10^6 asexual blood stages injected iv or intraperitoneally (ip) into five mice each respectively (data not shown). The fourth group (group IV) was challenged by infectious mosquito bite 45 and 210 days after the last immunization dose. All mice were protected when challenged with PyWT sporozoites and did not develop any blood-stage infection (Table 2). However, mice challenged with blood-stage parasites developed blood-stage parasitaemia after

Table 1. PySAP1-deficient sporozoites are completely attenuated and do not cause blood-stage infection in BALB/c mice.

| No. of injected sporozoites | Pysap1(−) | PyWT |
|----------------------------|-----------|------|
|                            | Infected  | Pre-patent period* | Infected  | Pre-patent period* |
| 20                         | ND        | ND   | 2/2        | 4 days   |
| 100                        | ND        | ND   | 6/6        | 4 days   |
| 10,000                     | 0/30      | –    | 8/8        | 3 days   |
| 100,000                    | 0/15      | –    | 3/3        | 2.5 days |
| 500,000                    | 0/8       | –    | ND         | ND       |
| 1,000,000                  | 0/4       | –    | ND         | ND       |
| > 2,000,000                | 0/3       | –    | ND         | ND       |

* The period (in days) between sporozoite infection and the detection of erythrocytic stages in blood smears. ND, not done.

Table 2. Immunization with Pysap1(−) sporozoites confers sterile protection against wild-type sporozoite challenge.

| Group | Primary dose (days of booster dose) | Challenge dose/ days after last boost | No. protected/ No. challenged* | Mean pre-patent period (days) |
|-------|------------------------------------|---------------------------------------|--------------------------------|-----------------------------|
| I     | 10,000 (14, 28)                    | 10,000/7                              | 9/9                            | –                           |
| II    | 10,000 (14, 28)                    | 10,000/0(30)/(210)                   | 15/15/15                      | –/–                         |
| III   | 10,000 (14, 28)                    | 10,000/0(30)/(210)                   | 10/10/10                      | –/–                         |
| IV    | 10,000 (14, 28)                    | MB/5(45)/(210)                       | 5/5/5                         | –/–                         |

* Each immunization group had an age-matched naive control group (minimum three mice) that all became patent at day 3 after each PyWT sporozoite challenge.

b. Infection through mosquito bite (MB) by allowing a minimum of 10 PyWT female infected mosquitoes, with midgut oocyst infectivity higher than 90%, to bite one mouse for at least 10 min.
2 days (data not shown). The data demonstrate that 
Pysap1(-) salivary gland sporozoite immunizations induce stage-specific sterile immunity against subsequent 
PyWT sporozoite infection but not against asexual blood-stage infection.

Pysap1(-) sporozoites traverse and invade hepatocytes normally but suffer an early liver-stage developmental arrest in vitro

Failure of mutant salivary gland sporozoites to induce blood-stage infection in mice can be due to distinct knock-out phenotypes (reviewed in Mikolajczak and Kappe, 2006). Pysap1(-) salivary gland sporozoites displayed continuous gliding motility that was undistinguishable from PyWT, tested on glass slides by direct microscopic examination (Vanderberg, 1974) (data not shown). Thereafter, we tested the cell-traversal capacity of Pysap1(-) salivary gland sporozoites using a cell-wounding assay (Vanderberg et al., 1990; Mota et al., 2001). Pysap1(-) sporozoites traversed hepatocytes and wounded cells at a rate comparable to PyWT sporozoites (Fig. 4A). In order to identify and characterize the deficiency of Pysap1(-) sporozoites in completing pre-erythrocytic infection, we conducted in vitro assays with the hepatoma cell line HepG2-CD81 which sustains productive P. yoelii sporozoite infection and liver-stage development (Silvie et al., 2006). Intrahepatocytic parasites were quantified by dif-

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd, Molecular Microbiology, 69, 152–163
Intracellular malaria parasites need a PVM for development (reviewed in Mikolajczak and Kappe, 2006). Therefore, we examined whether the observed lack of UIS4 in intracellular \textit{Pysap1(−)} parasites indicated a possible deficiency in PVM formation. We performed an electron microscopic analysis of intracellular WT and \textit{Pysap1(−)} parasites 1 h after infection of HepG2-CD81 cells. Intracellular \textit{Pysap1(−)} parasites were able to form a PVM (Fig. 5). Out of 15 intrahepatocytic \textit{Pysap1(−)} parasites evaluated by EM, 4 exhibited a PVM and 11 appeared free in the cytoplasm. The latter may represent sporozoites in the process of cell traversal. However, it is also possible that \textit{Pysap1(−)} sporozoites form a PVM but less efficiently than \textit{PyWT}.

\textbf{UIS gene products are depleted in \textit{Pysap1(−)} sporozoites}

Despite the presence of a PVM we noted the lack of \textit{PyUIS4} in \textit{Pysap1(−)} liver stages (Fig. 4C). \textit{PyUIS4} is normally expressed in sporozoite secretory organelles as well as the liver-stage PVM and is essential for malaria parasite liver-stage development (Mueller et al., 2005a,b). To test whether \textit{PyUIS4}, as well as other proteins, is expressed in \textit{Pysap1(−)} sporozoites prior to hepatocyte invasion, we performed IFAs to test UIS4, UIS3 and MTIP (Bergman et al., 2003) expression in \textit{Pysap1(−)} and \textit{PyWT} salivary gland sporozoites. In \textit{PyWT} sporozoites we detected expression of each protein (Fig. 6A). In contrast, we did not detect expression of UIS4 or UIS3 in \textit{Pysap1(−)} sporozoites but did detect MTIP staining (Fig. 6A). To test whether this expression pattern is due to a reduction in \textit{UIS4} and \textit{UIS3} transcript abundance, which would potentially indicate transcript degradation (Parker and Sheth, 2007), we performed RT-PCR analysis on \textit{Pysap1(−)} salivary gland sporozoite cDNA. Strikingly, we observed a severe reduction of \textit{PyUIS4} and \textit{PyUIS3} (Mueller et al., 2005a,b) transcript abundance (Fig. 6B and C). Furthermore, we saw a decrease in \textit{P52} (van Dijk et al., 2005; Ishino et al., 2005a,b; Labaied et al., 2007) transcript abundance as well as the transcripts of two uncharacterized UIS genes, \textit{UIS2} (putative secreted phosphatase) and \textit{UIS28} (putative secreted lipase).
Conversely, transcript abundance for genes that are involved in sporozoite functions prior to PVM formation and liver-stage development appeared not significantly reduced in \( Pysap1(\cdot) \) sporozoites (Fig. 6B and C). These genes included CSP, TRAP (reviewed in Kappe et al., 2004; Baldacci and Menard, 2004), SPECT1 (Ishino et al., 2004), SPECT2 (Ishino et al., 2005b) and S4/CELTOS (Kaiser et al., 2004, Kariu et al., 2006). Therefore, lack of \( Py\text{-SAP1} \) in sporozoites has a selective negative impact on UIS gene expression in \( P. yoelii \).

**Discussion**

Successful hepatocyte infection and liver-stage development by the malaria parasite is dependent on establishment of a PVM as the functional host–parasite interface. Some UIS gene products uniquely expressed in salivary gland sporozoites and localized to the PVM in liver stages play essential roles in intrahepatocytic parasite survival (Mueller et al., 2005a,b; Mikolajczak et al., 2007a; Tarun et al., 2007). However, factors that allow salivary gland sporozoites lying-in-wait in the mosquito salivary glands to initiate a co-ordinated switch to mammalian host infection by differential expression of UIS have not been identified. Our work identifies SAP1 as such a potential factor. \( Py\text{-SAP1} \) is the first identified cytoplasmic \( Plasmodium \) protein with an essential function for pre-erythrocytic stages. It has a large internal asparagine-rich low-complexity domain. Low-complexity domains in \( Plasmodium \) proteins were hypothesized to be an evolutionary by-product with no significant function in the biology of the malaria parasite (Xue and Forsdyke, 2003). In contrast, low-complexity proteins have been
proposed as virulence inducing factors in some pathogenic bacterial strains (Nandi et al., 2003). The low-complexity domain of SAP1 is flanked by two highly conserved non-asparagine-rich N- and C-terminal domains. The level of conservation in these domains is high among SAP1 proteins from distinct Plasmodium species, indicating that they represent functionally important regions. Pysap1(−) salivary gland sporozoites show extremely reduced transcript abundance for UIS3, UIS4 and P52 but not SPECTs, TRAP and CSP, indicating a selective mechanism of UIS transcript depletion. It will be interesting to determine whether transcript abundance for additional genes is affected in the Pysap1(−) sporozoites as we have shown here for the uncharacterized UIS2 and UIS28. Clearly, lack of UIS genes expression cannot be attributed to a defect in salivary gland invasion and residence as Pysap1(−) sporozoites infected the salivary glands with efficiencies that are comparable to WT sporozoites. Therefore, it appears likely that the reduction of UIS transcript expression in Pysap1(−) sporozoites is a direct effect of the lack of SAP1 and not an indirect effect of an altered biological behaviour of the Pysap1(−) mutants. Transcript abundance in eukaryotes is mainly regulated by transcriptional and post-transcriptional mechanisms. PySAP1 localization to the sporozoite cytoplasm and absence from the sporozoite nucleus suggests that PySAP1 is involved in as-yet-to-be-defined post-transcriptional mechanisms of UIS transcript regulation, as post-transcriptional regulation is expected to be executed in the cytoplasm of the cell (Elemento et al., 2007; Parker and Sheth, 2007). However, the C-terminus of PySAP1, which is recognized by the antisera, might be processed and left in the cytoplasm. In this scenario, the remaining part of the protein might translocate to the nucleus, where it might exert its effect by contributing to the regulation of transcription of UIS genes. Interestingly, post-transcriptional, but not transcriptional, regulation has been hypothesized to be the main pathway for controlling the expression levels of proteins in Plasmodium (Coulson et al., 2004; Hakimi and Deitsch, 2007). Indeed, it has been suggested that Plasmodium must rely on this mechanism for controlling the extensive differential gene expression required during the complex malaria parasite life cycle (Hall et al., 2005). Recently, it has been shown that a RNA helicase termed DOZI (Development of Zygote Inhibited) is expressed in the female gametocyte where it localizes to cytoplasmic protein complexes and is involved in translational repression of transcripts (Mair et al., 2006). DOZI knockouts showed a severe reduction in the levels of many sexual stage-specific transcripts, presumably because they were subject to rapid degradation when not protected in ribonucleoprotein (RNP) complexes. We speculate that SAP1 might be an essential component of such an RNP complex in sporozoites and protects UIS transcripts specifically from degradation. This scenario however requires further investigation. Interestingly, proteins with glutamine and asparagine-rich domains have recently been shown to be part of RNP complexes in yeast where they act as scaffolding proteins (Decker et al., 2007).

Pysap1(−) sporozoites showed complete attenuation of liver infection. This is clearly attributable to the lack of the essential proteins UIS3, UIS4 and P52 and possibly additional UIS in the knockout parasite and likely not to a lack of a direct effect function of PySAP1. Single- and double-gene-deletion sporozoites are effective live attenuated vaccines in mouse models (reviewed in Mikolajczak et al., 2007b) and we have shown herein that Pysap1(−) sporozoites also confer sterile long-lasting protection against PyWT sporozoite challenge. P. falciparum gene deletion mutants might go forward for testing as human malaria vaccines (Renia et al., 2006). We suggest that a putative P. falciparum sap1(−) sporozoite may be an attractive live attenuated vaccine candidate due to its quasi-multilocus attenuation. Together, our data give initial insights into the regulation of malaria parasite infectivity after mosquito transmission and these findings might advance efforts to develop measures for prevention of malaria infection.

Experimental procedures

Experimental animals, parasites and cell lines

Six- to 8-week-old female BALB/cJ (for in vivo infection studies and immunizations) or Swiss Webster (SW) mice (for parasite cycle maintenance) were purchased from the Jackson Laboratory (Bar Harbor, ME) or Harlan (Indianapolis, IN). Animal handling was conducted according to Institutional Animal Care and Use Committee-approved protocols. Wild-type P. yoelii 17XLN (non-lethal strain) clone 1.1 (Weiss et al., 1989 ) and Pysap1(−) parasites were cycled between SW mice and Anopheles stephensi mosquitoes. Infected mosquitoes were maintained on sugar water at 24°C and 70% humidity. Salivary gland sporozoites were extracted from infected mosquitoes between days 13 and 15 post blood meal infection as described before (Labaied et al., 2007; Tarun et al., 2007). The human hepatoma cell line HepG2-CD81 (Silvie et al., 2006) was used for all in vitro assays and was maintained in DMEM-F12 medium supplemented with antibiotics and 10% fetal calf serum (FCS).

Generation of Pysap1(−) parasites

Targeted deletion of PySAP1 by double-cross-over homologous recombination was achieved constructing a replacement plasmid in the b3D.DT.H Db targeting vector (Janse et al., 2006; Jongco et al., 2006). P. yoelii 17XLN genomic DNA (gDNA) was used as a template to amplify a 1.5 kb fragment of the 5′UTR of PySAP1 using oligonucleotide primers PySAP1rep1 forward (F) and PySAP1rep2 reverse
Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with TURBO DNase (Ambion) from *P. yoelii* salivary gland sporozoites (2 × 10⁶), oocyst sporozoites (2 × 10⁶) or mixed blood stages (1 × 10⁷ infected red blood cells were isolated from an infected mouse by heart puncture bleeding and serial limiting dilution of infected blood). *P. falciparum* sporozoites and mixed blood-stage RNA materials were kindly provided by Dr Urszula Krzych and Dr Jack Williams (WRAIR, Silver Spring, MD). cDNA synthesis was performed using the Super Script III Platinum two-step qRT-PCR kit (Invitrogen). The sequences of specific primers used for amplification from cDNA are listed in Table S2. All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation, 55°C for 30 s for primer annealing, 60°C for 4 min for DNA strands extension.

Immunofluorescence assays (IFAs)

We generated rabbit polyclonal antiserum against a *PySAP1*_{3020-3034} synthetic peptide (LRGROVQOSFNHSAS). The antiserum was further affinity-purified against the synthetic peptide and the specific IgGs were concentrated. *PySap1*−/− or *PyWT* sporozoites were air dried on poly-l-lysine coated chamber slides at a density of 60 000 cells well−1 and treated with TURBO DNase (Ambion) from *P. yoelii* salivary gland sporozoites (2 × 10⁶), oocyst sporozoites (2 × 10⁶) or mixed blood stages (1 × 10⁷ infected red blood cells were isolated from an infected mouse by heart puncture bleeding and serial limiting dilution of infected blood). *P. falciparum* sporozoites and mixed blood-stage RNA materials were kindly provided by Dr Urszula Krzych and Dr Jack Williams (WRAIR, Silver Spring, MD). cDNA synthesis was performed using the Super Script III Platinum two-step qRT-PCR kit (Invitrogen). The sequences of specific primers used for amplification from cDNA are listed in Table S2. All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation, 55°C for 30 s for primer annealing, 60°C for 4 min for DNA strands extension.

Cell-traversal assay

Hepatoma HepG2-CD81 cells were inoculated in eight-well chamber slides at a density of 60 000 cells well−1 and treated with TURBO DNase (Ambion) from *P. yoelii* salivary gland sporozoites (2 × 10⁶), oocyst sporozoites (2 × 10⁶) or mixed blood stages (1 × 10⁷ infected red blood cells were isolated from an infected mouse by heart puncture bleeding and serial limiting dilution of infected blood). *P. falciparum* sporozoites and mixed blood-stage RNA materials were kindly provided by Dr Urszula Krzych and Dr Jack Williams (WRAIR, Silver Spring, MD). cDNA synthesis was performed using the Super Script III Platinum two-step qRT-PCR kit (Invitrogen). The sequences of specific primers used for amplification from cDNA are listed in Table S2. All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation, 55°C for 30 s for primer annealing, 60°C for 4 min for DNA strands extension.

Mouse infections and immunizations

For sporozoite immunizations and challenges, BALB/cJ mice were injected iv with sporozoites re-suspended in incomplete DMEM-F12 medium. Blood-stage patency was monitored daily by evaluation of Giemsa-stained blood smears from day 2 to day 14 post sporozoite infection. For blood-stage challenge experiments, immunized mice that had been challenged with sporozoites twice were injected either ip with 10⁶ or iv with 10³*PyWT* erythrocytic asexual stages in RPMI. *PyWT* blood stages were isolated from an infected mouse by heart puncture followed by serial limited dilution.

In vitro infection assays

We standardized a differential permeabilization hepatoma infection assay to specifically quantify liver-stages parasites at different time points of infection by fluorescence microscopy. Hepatoma HepG2-CD81 cells were seeded in eight-well chamber slides at a density of 40 000−50 000 cells well−1 and treated with TURBO DNase (Ambion) from *P. yoelii* salivary gland sporozoites (2 × 10⁶), oocyst sporozoites (2 × 10⁶) or mixed blood stages (1 × 10⁷ infected red blood cells were isolated from an infected mouse by heart puncture bleeding and serial limiting dilution of infected blood). *P. falciparum* sporozoites and mixed blood-stage RNA materials were kindly provided by Dr Urszula Krzych and Dr Jack Williams (WRAIR, Silver Spring, MD). cDNA synthesis was performed using the Super Script III Platinum two-step qRT-PCR kit (Invitrogen). The sequences of specific primers used for amplification from cDNA are listed in Table S2. All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation, 55°C for 30 s for primer annealing, 60°C for 4 min for DNA strands extension.
non-invading and unbound sporozoites and mosquito debris. One hour post infection assays were fixed with 4% PFA (which does not permeabilize hepatocytes) for 10 min at room temperature, followed by permeabilization (or not) with ice-cold methanol for 5 min at room temperature and then blocking in 10% FCS/PBS (ON) at 4°C. The cells for other post-infection time points assays were further completed in complete DMEM medium until fixed, permeabilized (or not) and blocked at 6 h, 12 h, 18 h and 24 h. Primary antibodies against PyCSP, PyHSP70 and PyUIS4 were diluted to 1:300 in 10% FCS/PBS and incubated with the cells for 1 h at 37°C. Conjugated secondary anti-mouse Alexa Fluor 488 (green) and anti-rabbit Alexa Fluor 594 (red) were used to visualize the bound primary antibodies. Nuclear staining with DAPI (1:3000 diluted) was performed with the last wash and before mounting the slides with an antifade reagent. Preparations were analysed using fluorescence confocal microscopy (Olympus 1 × 10 Delta Vision). Intracellular parasites were determined as the total number of parasites counted in each permeabilized sample well as a fraction of the total number of parasites counted in the control unpermeabilized sample well.

Transmission electron microscopy

For thin-section transmission electron microscopy, 10⁶ PyWT and Pysap1(−) sporozoites were used to infect 10⁸ subconfluent HepG2-CD81 cells. One hour post infection, cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and processed as described previously (Quittnat et al., 2004), before examination with a Philips 410 electron microscope (Eindhoven, the Netherlands) under 80 kV.

Acknowledgements

We thank Dr Urszula Krzych and Dr Jack Williams for providing us with P. falciparum sporozoites and blood-stages materials. We also thank Xinxia Peng, Sasha DeLeon and Ronald Dumpit for excellent technical assistance and Dr Ashley Vaughan for critically reading this manuscript. This work was funded by a Grant from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative.

References

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006) Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 12: 220–224.

Aravind, L., Iyer, L.M., Wellems, T.E., and Miller, L.H. (2003) Plasmodium biology: genomic gleanings. Cell 115: 771–785.

Baldačci, P., and Menard, R. (2004) The elusive malaria sporozoite in the mammalian host. Mol Microbiol 54: 298–306.

Belmonte, M., Jones, T.R., Lu, M., Arcilla, R., Smalls, T., Belmonte, A., et al. (2003) The infectivity of Plasmodium yoelii in different strains of mice. J Parasitol 89: 602–603.

Bergman, L.W., Kaiser, K., Fujikoa, H., Coppens, I., Daly, T.M., Fox, S., et al. (2003) Myosin A tail domain interacting protein (MTIP) localizes to the inner membrane complex of Plasmodium sporozoites. J Cell Sci 116: 39–49.

Carlton, J.M., Angiuoli, S.V., Suh, B.B., Kooji, T.W., Pertea, M., Silva, J.C., et al. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii. Nature 419: 512–519.

Coulson, R.M., Hall, N., and Ouzounis, C.A. (2004) Comparative genomics of transcriptional control in the human malaria parasite Plasmodium falciparum. Genome Res 14: 1548–1554.

Decker, C.J., Teixeira, D., and Parker, R. (2007) Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in Saccharomyces cerevisiae. J Cell Biol 179: 437–449.

van Dijk, M.R., Douradinha, B., Franke-Fayard, B., Heussler, V., van Dooren, M.W., van Schaikj, B., et al. (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. Proc Natl Acad Sci USA 102: 12194–12199.

Elemento, O., Slonim, N., and Tavazoie, S. (2007) A universal framework for regulatory element discovery across all genomes and data types. Mol Cell 28: 337–350.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419: 498–511.

Hakimi, M.A., and Deitsch, K.W. (2007) Epigenetics in Apicomplexa: control of gene expression during cell cycle progression, differentiation and antigenic variation. Curr Opin Microbiol 10: 357–362.

Hall, N., Karras, M., Raine, J.D., Carlton, J.M., Kooji, T.W., Berriman, M., et al. (2005) A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science 307: 82–86.

Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2004) Cell passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. PLoS Biol 2: E4.

Ishino, T., Chinzei, Y., and Yuda, M. (2005a) Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. Mol Microbiol 58: 1264–1275.

Ishino, T., Chinzei, Y., and Yuda, M. (2005b) A Plasmodium sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. Cell Microbiol 7: 199–208.

Janse, C.J., Franke-Fayard, B., Mair, G.R., Ramesar, J., Thiel, C., Engelmann, S., et al. (2006) High efficiency transfection of Plasmodium berghei facilitates novel selection procedures. Mol Biochem Parasitol 145: 60–70.

Jongco, A.M., Ting, L.M., Thathy, V., Mota, M.M., and Kim, K. (2006) Improved transfection and new selectable markers for the rodent malaria parasite Plasmodium yoelii. Mol Biochem Parasitol 146: 242–250.

Kaiser, K., Matuschewski, K., Camargo, N., Ross, J., and Kappe, S.H. (2004) Differential transcriptome profiling identifies Plasmodium genes encoding pre-erythrocytic stage-specific proteins. Mol Microbiol 51: 1221–1232.

Kappe, S.H., Buscaglia, C.A., and Nussenzweig, V. (2004) Plasmodium sporozoite molecular cell biology. Annu Rev Cell Dev Biol 20: 29–59.
Kariu, T., Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2006) Cetitos, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. Mol Microbiol 59: 1369–1379.

Labadie, M., Harupa, A., Dumpit, R.F., Coppen, I., Mikolajczak, S.A., and Cape, S.H. (2007) Plasmodium yoelli sporozoites with simultaneous deletion of PS2 and PS6 are completely attenuated and confer sterile immunity against infection. Infect Immun 75: 3758–3768.

Mair, G.R., Braks, J.A., Garver, L.S., Wiegant, J.C., Hall, N., Dirks, R.W., et al. (2006) Regulation of sexual development of Plasmodium by transcriptional repression. Science 313: 667–669.

Matuschewski, K. (2006) Getting infectious: formation and maturation of Plasmodium sporozoites in the Anopheles vector. Cell Microbiol 8: 1547–1556.

Matuschewski, K., Ross, J., Brown, S.M., Kaiser, K., Nussenzweig, V., and Cape, S.H. (2002) Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. J Biol Chem 277: 41948–41953.

Menard, R., and Janse, C. (1997) Gene targeting in malaria parasites. Methods 13: 148–157.

Mikolajczak, S.A., and Cape, S.H. (2006) A clash to conquer: the malaria parasite liver infection. Mol Microbiol 62: 1499–1506.

Mikolajczak, S.A., Jacobs-Lorena, V., MacKellar, D.C., Camargo, N., and Cape, S.H. (2007a) L-FABP is a critical host factor for successful malaria liver stage development. Int J Parasitol 37: 483–489.

Mikolajczak, S.A., Aly, A.S., and Cape, S.H. (2007b) Preerythrocytic malaria vaccine development. Curr Opin Infect Dis 20: 461–466.

Mota, M.M., Pradel, G., Vanderberg, J.P., Hafalla, J.C., Frevert, U., Nussenzweig, R.S., et al. (2001) Migration of Plasmodium sporozoites through cells before infection. Science 291: 141–144.

Mueller, A.K., Labadie, M., Cape, S.H., and Matuschewski, K. (2005a) Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. Nature 433: 164–167.

Mueller, A.K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., and Cape, S.H. (2005b) Plasmodium liver stage developmental arrest by depletion of a protein at the parasite–host interface. Proc Natl Acad Sci USA 102: 3022–3027.

Nandi, T., Kannan, K., and Ramachandran, S. (2003) The low complexity proteins from enteric pathogenic bacteria: taxonomic parallels embedded in diversity. In Silico Biol 3: 277–285.

Parker, R., and Sheth, U. (2007) P bodies and the control of mRNA translation and degradation. Mol Cell 25: 635–646.

Pizzi, E., and Frontali, C. (2001) Low-complexity regions in Plasmodium falciparum proteins. Genome Res 11: 218–229.

Potocnjak, P., Yoshida, N., Nussenzweig, R.S., and Nussenzweig, V. (1980) Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (PB44) protect mice against malarial infection. J Exp Med 151: 1504–1513.

Quittnat, F., Nishikawa, Y., Stedman, T.T., Voelker, D.R., Choi, J.Y., Zahn, M.M., et al. (2004) On the biogenesis of lipid bodies in ancient eukaryotes: synthesis of triacylglycerols by a Toxoplasma DGAT1-related enzyme. Mol Biochem Parasitol 138: 107–122.

Renia, L., Gruner, A.C., Mauduit, M., and Snounou, G. (2006) Vaccination against malaria with live parasites. Expert Rev Vaccines 5: 473–481.

Silvie, O., Greco, C., Franetich, J.F., Dubart-Kupperschmitt, A., Hannoun, L., van Gemert, G.J., et al. (2006) Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the Plasmodium species. Cell Microbiol 8: 1134–1146.

Singh, G.P., Chandra, B.R., Bhattacharya, A., Akhouri, R.R., Singh, S.K., and Sharma, A. (2004) Hyper-expansion of asparagines correlates with an abundance of proteins with prion-like domains in Plasmodium falciparum. Mol Biochem Parasitol 137: 307–319.

Tarun, A.S., Dumpit, R.F., Camargo, N., Labadie, M., Liu, P., Takagi, A., et al. (2007) Protracted sterile protection with Plasmodium yoelli pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells. J Infect Dis 196: 608–616.

Tsujii, M., Mattei, D., Nussenzweig, R.S., Eichinger, D., and Zavala, F. (1994) Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites. Parasitol Res 80: 16–21.

Vanderberg, J.P. (1974) Studies on the motility of Plasmodium sporozoites. J Protozool 21: 527–537.

Vanderberg, J.P. (1975) Development of infectivity by the Plasmodium berghei sporozoite. J Parasitol 61: 43–50.

Vanderberg, J.P., and Frevert, U. (2004) Intravitral microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. Int J Parasitol 34: 991–996.

Vanderberg, J.P., Chew, S., and Stewart, M.J. (1990) Plasmodium sporozoite interactions with macrophages in vitro: a videomicroscopic analysis. J Protozool 37: 528–536.

Weiss, W.R., Good, M.F., Hollingdale, M.R., Miller, L.H., and Berzofsky, J.A. (1989) Genetic control of immunity to Plasmodium yoelli sporozoites. J Immunol 143: 4263–4266.

Xue, H.Y., and Forsdyke, D.R. (2003) Low-complexity regions of Plasmodium falciparum proteins are primarily nucleic acid level adaptations. Mol Biochem Parasitol 128: 21–32.

Yoshida, N., Nussenzweig, R.S., Potocnjak, P., Nussenzweig, V., and Akaiwa, M. (1980) Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. Science 207: 71–73.

Supplementary material

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06271.x

(This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd, Molecular Microbiology, 69, 152–163