The Puf-Family RNA-Binding Protein Puf2 Controls Sporozoite Conversion to Liver Stages in the Malaria Parasite

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Abstract

Malaria is a vector-borne infectious disease caused by unicellular, obligate intracellular parasites of the genus *Plasmodium*. During host switch the malaria parasite employs specialized latent stages that colonize the new host environment. Previous work has established that gametocytes, sexually differentiated stages that are taken up by the mosquito vector, control expression of genes required for mosquito colonization by translational repression. Sexual parasite development is controlled by a DEAD-box RNA helicase of the DDX6 family, termed DOZI. Latency of sporozoites, the transmission stage injected during an infectious blood meal, is controlled by the elf2alpha kinase IK2, a general inhibitor of protein synthesis. Whether RNA-binding proteins participate in translational regulation in sporozoites remains to be studied. Here, we investigated the roles of two RNA-binding proteins of the Puf-family, *Plasmodium* Puf1 and Puf2, during sporozoite stage conversion. Our data reveal that, in the rodent malaria parasite *P. berghei*, Puf2 participates in the regulation of IK2 and inhibits premature sporozoite transformation. Inside mosquito salivary glands puf2(-) sporozoites transform over time to round forms resembling early intra-hepatic stages. As a result, mutant parasites display strong defects in initiating a malaria infection. In contrast, Puf1 is dispensable in vivo throughout the entire *Plasmodium* life cycle. Our findings support the notion of a central role for Puf2 in parasite latency during switch between the insect and mammalian hosts.

Introduction

*Plasmodium* parasites, the causative agents of malaria, are transmitted by female *Anopheles* mosquitoes. During the probing phase prior to the blood meal, sporozoites are injected into the skin of the mammalian host [1]. The motile sporozoites actively migrate in the skin, enter the peripheral blood circulation, and then rapidly reach the liver. Sporozoites invade hepatocytes by forming a parasitophorous vacuole (PV) [2], where they transform into replicative exo-erythrocytic forms (EEFs). After intense multiplication during 2–6 days, depending on the *Plasmodium* species, mature EEFs release thousands of merozoites, which invade erythrocytes and initiate the pathogenic blood stage cycle [3].

*Plasmodium* sporozoites are formed inside oocytes in the mosquito midgut, but become fully infective only after colonization of the insect salivary glands. This maturation process is associated with the up-regulation of a specific subset of genes, referred to as UP-regulated in Infective Sporozoites (UIS) genes [4]. Regulation of gene expression in *Plasmodium* remains poorly understood. Genome sequencing data initially revealed a paucity of specific transcription factors in *Plasmodium* [5]. Recently, however, a family of genes related to the plant Apetala-2 (AP2) transcription factors has been identified in *Plasmodium* and related apicomplexan parasites [6,7], and proposed to play a central role during life cycle progression. Molecular genetic studies have demonstrated vital roles of two stage-specific AP2 factors in *Plasmodium berghei*, a rodent malaria parasite widely used as a model [8,9]. One of these factors, the AP2-Sp transcription factor, is required during sporozoite differentiation and binds to a specific DNA sequence found in the promoter region of many genes expressed in sporozoites, including, but not restricted to, UIS genes [8]. Intriguingly, genes containing AP2-Sp binding sites are associated with a wide range of biological processes, such as sporozoite formation, host cell invasion or liver stage development. This observation strongly suggests that additional mechanisms participate in the fine-tuning of gene expression during sporozoite development and stage conversion. Another factor, called SLARP or SAP1, controls the expression of a subset of genes in sporozoites, and plays a critical role during intrahepatic development of the parasite [10,11]. It is still unclear whether SLARP/SAP1 acts on a transcriptional or a post-transcriptional level. The cellular localization of SLARP/SAP1 remains controversial [10,11], and the absence of any domain known to bind nucleic acids suggests an indirect role.

More recently, Zhang and colleagues reported that the protein kinase IK2, initially termed UIS1 [4], controls global gene expression in sporozoites at a post-transcriptional level [12]. IK2...
phosphorylates the translation initiation factor eIF2alpha and down-regulates protein synthesis [12,13]. P. berghei lacking UIS1/IK2 display a partial loss of infectivity associated with premature transformation of sporozoites in the mosquito salivary glands [12]. The contribution of RNA-binding proteins in translational regulation has not been studied in sporozoites yet, but has been well characterized in Plasmodium sexual stages. In female gametocytes, many transcripts encoding ookinete proteins are translationally repressed by a DEAD-box RNA helicase called DOZI, which binds to the 3' untranslated region (UTR) of target mRNAs such as P28 and blocks their translation until occurrence of gamete fertilization and differentiation into a zygote and ookinete [14,15]. Whether DOZI plays a role in sporozoites is not known, but other RNA-binding proteins may participate in translational regulation in sporozoites, including members of the Puf-family.

Puf proteins are evolutionary conserved in eukaryotes and are characterized by the presence of a RNA-binding Puf domain, named after the Drosophila melanogaster protein Pumilio and the Caenorhabditis elegans protein fmr-3 binding factor (FBF), and consisting of eight imperfect repeats of 36 amino acids (PFAM:PF00806) [16,17]. Puf proteins typically bind to the 3' UTR of target mRNAs and repress their translation or induce their degradation (reviewed in [18] and [19]). Plasmodium parasites possess two genes encoding proteins with Puf domains, Puf1 and Puf2 [20]. In P. falciparum, both Puf1 (PFE0935c) and Puf2 ( PfD0825c) are differentially expressed in gametocytes [20,21]. Targeted gene disruption in P. falciparum recently revealed a role of PfPuf2 in repressing gametocytogenesis and male gametocyte differentiation in the human malaria parasite [22]. Whether the Puf2 protein plays additional, perhaps vital, roles in subsequent life cycle stages remains to be shown. Interestingly, microarray data indicate that Puf2 is most highly expressed in P. falciparum sporozoites [23], and in P. berghei, expression of both Puf1 (PBANKA_123350) and Puf2 (PBANKA_071920) has been reported in sporozoites, where Puf1 was initially identified as UIS9 [4,24]. In this study, we used a reverse genetic approach to investigate the roles of Puf1 and Puf2 in P. berghei, with the aim to identify potential mRNA binding proteins that play critical roles in sporozoite stage conversion.

Results

Targeted gene deletion of P. berghei Puf1 and Puf2

We first assessed the expression of Puf1 and Puf2 during P. berghei development in the insect vector, in comparison to DOZI and UIS1/IK2, using quantitative RT-PCR (Figure 1). Similarly to UIS1/IK2 [12], we found that Puf1 and Puf2 are upregulated in P. berghei salivary gland sporozoites (Figure 1). This was expected for Puf1, which was initially described as UIS9 [4,24]. Furthermore, Puf1 was also upregulated in gametocytes and ookinetes, similarly to IK2 and DOZI. In good agreement with published microarray data [24], only low levels of DOZI mRNA were detected in P. berghei sporozoites (Figure 1). In contrast to Puf1 and Puf2, DOZI steady state mRNA levels were down-regulated in infectious salivary gland-associated sporozoites resulting in ~100 fold lower levels in the latent transmission stage. Together, the expression profiling indicated that both Puf members could play a role in sporozoite stage conversion, as has been described previously for the eIF2alpha kinase UIS1/IK2 [12].

In order to investigate the functional importance of Puf1/UIS9 and Puf2 in P. berghei, we generated loss-of-function mutants (Figure 2). We used a replacement strategy to disrupt the endogenous Puf1 (Figure 2A) or Puf2 (Figure 2B) gene copy by double crossover homologous recombination [25]. Targeting constructs containing 3' and 3' fragments of either Puf1 or Puf2 flank ing a pyrimethamine-resistance cassette were used to transfect P. berghei parasites that constitutively express GFP (ANKA cl507) [26]. Recombinant parasites were selected with pyrimethamine in the mouse drinking water, and cloned by limiting dilutions. For both genes we were successful in generating clonal knockout parasite populations, as demonstrated by PCR and Southern blot analysis of genomic DNA (Figures 2C-F). For Puf2 we also generated a second independent knockout clone, which was phenotypically identical to the first puf2(-) clonal parasite line (unpublished data). This indicates that Puf1 and Puf2 do not play any vital role during the sexual development of P. berghei erythrocytic stages, in good agreement with successful generation of Ppuf2(-) parasites [22].

puf1(-) and puf2(-) parasites produce gametocytes that develop to sporozoites in mosquitoes

puf1(-) and puf2(-) parasites are indistinguishable from WT parasites in development and growth of asexual blood stages and produced gametocytes. Because PfPuf2 has been shown to control gametocytogenesis in P. falciparum [22], we analyzed in more detail the sexual development of P. berghei puf2(-) parasites. After injection of 10⁷ infected erythrocytes intravenously into groups of five C57BL/6 mice, parasitemia at day 4 were similar in mice infected with WT or puf2(-) parasites (Figure 3A). However, the proportion of gametocytes among all parasite stages was significantly higher in puf2(-) than in WT parasites (Figure 3B). We then examined the ability of mature male gametocytes to exflagellate in puf2(-) parasites. The number of exflagellation centers in mouse blood was significantly higher for puf2(-) parasites than for WT parasites (Figure 3C), suggesting that male gametocytes contribute to the increased gametocytogenesis in Ppuf2(-) parasites, in full support of the data reported for P. falciparum PfPuf2-deficient parasites [22]. After transmission to Anopheles stephensi mosquitoes, both puf1(-) and puf2(-) parasite lines produced oocysts and high numbers of
sporozoites (Table 1). The number of puf2(-) oocysts was significantly higher than for WT, consistent with the higher gametocyte rates. Intriguingly, we found lower numbers of oocysts and salivary gland sporozoites in puf1(-)-infected mosquitoes, as compared to WT parasites (Table 1). Although the differences were not statistically significant, we cannot exclude an effect of puf1 depletion on oocyst development and sporogony.

Liver infection is impaired in Puf2-deficient parasites

We then analysed the infectivity of puf1(-) and puf2(-) sporozoites to susceptible mice. C57BL/6 mice were injected intravenously with 1,000 WT, puf1(-) or puf2(-) P. berghei sporozoites, or exposed to the bites of 10 infected mosquitoes, the natural transmission route (Table 1). Emergence of erythrocytic stages, resulting from complete liver stage development, was monitored by microscopic examination of daily blood smears. With both inoculation routes, all mice injected with puf1(-) sporozoites developed a parasitemia, with no delay as compared to WT parasites (Table 1). In contrast, only a fraction of the mice injected with puf2(-) sporozoites developed a parasitemia, with a two-day delay as compared to WT, indicative of at least 100-fold reduction of infectivity (Table 1). Moreover, puf2(-) sporozoites isolated late after mosquito infection (at day 25) were not capable of inducing a blood stage infection in mice.

Table 1. Loss of infectivity of puf2(-) sporozoites in C57BL/6 mice.

| Parasites | Number of oocysts/mosquito* | Number of salivary gland sporozoites/mosquito* | Route of injection | Number of infected/Number of injected | Prepatency period (days)* |
|-----------|-----------------------------|-----------------------------------------------|-------------------|--------------------------------------|--------------------------|
| WT        | 182 (± 164)                 | 31,600 (± 18,600)                            | bites (d 21)      | 3/3                                  | 3                        |
|           |                             |                                               | i.v. (d 18)       | 2/2                                  | 3                        |
|           |                             |                                               | i.v. (d 21)       | 6/6                                  | 3.5                      |
|           |                             |                                               | i.v. (d 25)       | 4/4                                  | 3                        |
| puf1(-)   | 137 (± 100)                 | 11,400 (± 4,700)                            | bites (d 17)      | 3/3                                  | 3                        |
|           |                             |                                               | i.v. (d 25)       | 4/4                                  | 3                        |
| puf2(-)   | 320 (± 234)                 | 25,000 (± 18,800)                           | bites (d 21)      | 3/3                                  | 3                        |
|           |                             |                                               | i.v. (d 18)       | 2/4                                  | 3                        |
|           |                             |                                               | i.v. (d 25)       | 0/4                                  | NA                      |

*The number of midgut oocysts and salivary gland sporozoites was determined at d10-14 and d18-25, respectively, after the infectious blood meal, from at least three independent feeding experiments.

**C57BL/6 mice were exposed to the bites of 10 infected mosquitoes or injected intravenously (i.v.) with 1,000 sporozoites, 18-25 days after mosquito infection.

The prepatent period is defined as the number of days after sporozoite inoculation until detection of infected erythrocytes by microscopic blood smear examination. Brackets indicate that not all animals became infected. NA, not applicable.

*p<0.05 in comparison to WT, as determined by Kruskal-Wallis followed by Dunn's test.

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We next injected C57BL/6 mice intravenously with WT, puf1(-) or puf2(-) sporozoites isolated on day 18 from mosquito salivary glands. Forty-two hours after infection, livers were removed and the parasite loads were quantified by RT-qPCR. As shown in Figure 4, the puf2(-) liver loads were extremely reduced (~500 fold) as compared to WT, confirming that infectivity of puf2(-) sporozoites to C57BL/6 mice is severely impaired. The reduction of parasite liver loads as measured by RT-qPCR is consistent with the delay or absence of parasitemia in mice injected with puf2(-) sporozoites (Table 1), therefore we assume that the absence of Puf2 did not interfere with 18S rRNA quantification. Interestingly, we also observed a significant, although less pronounced (~4 fold), reduction of puf1(-) parasite liver loads (Figure 4). Our findings demonstrate that PbPuf2 plays an important in vivo role only in the pre-erythrocytic phase of the Plasmodium life cycle. In contrast, PbPuf1/UIS9 appears to be dispensable for parasite life cycle progression, at least under the conditions tested.

We also determined the In vitro infectivity of puf1(-) and puf2(-) sporozoites isolated on day 22 from mosquito salivary glands, in cultured HepG2 hepatoma cells (Figure 5). Both puf1(-) and puf2(-) sporozoites entered hepatoma cells as efficiently as WT, as evidenced by similar numbers of infected cells at early time points (4-6 hours) (Figure 5A). While the number of EEJs at later time...
points (24–48 hours) was similar in WT- and puf1(-)-infected cultures (Figure 5A), it was reduced in the case of puf2(-) parasites (Figure 5B). Whereas early after infection a vast majority (81% ± 3%; n = 122) of intracellular WT sporozoites expressed UIS4, a transmembrane protein that localizes to the membrane of the PV [27], only half of puf2(-) parasites were stained with UIS4 antibodies (53% ± 9%; n = 127). This indicates that a substantial fraction of puf2(-) sporozoites failed to form and/or remodel the PV in vitro, which probably explains the reduced EEF numbers quantified at later time points. In addition, we cannot exclude a moderate impairment during liver stage development in puf2(-) parasites, as suggested by the reduction of EEF numbers observed between 24 and 48 hours post-infection in vitro. Nevertheless, most puf2(-) sporozoites that formed a PV and expressed UIS4 were capable of developing into EEFs like WT and puf1(-) parasites (Figure 5C). Taken together, our data indicate that Puf2 plays a critical role during transmission of P. berghei sporozoites to the mammalian host, but is not required for liver stage development per se.

puf2(-) sporozoites transform prematurely in the mosquito

In vivo data suggested that, over time, Puf2-knockout sporozoites rapidly lose infectivity in the mosquito (Table 1). To better characterize this phenomenon, we carefully analyzed puf2(-) sporozoite development in the mosquito (Figure 6). Strikingly, we observed that a major proportion of puf2(-) sporozoites showed signs of premature transformation, characterized by a bulb-like aspect or even complete rounding-up (Figure 6A). In WT parasites, transformation of sporozoites is typically observed at 37°C in culture medium, irrespective of the presence of host cells [28]. In puf2(-)-infected mosquitoes, however, the proportion of transformed sporozoites increased over time during the course of infection in the mosquitoes, which are kept at 20°C (Figure 6B). Quantification of partial and complete transformation in all three parasite populations revealed that at day 29 almost all puf2(-)

Figure 3. Gametocytogenesis is increased in puf2(-) parasites. Groups of C57BL/6 mice (n = 5) were injected intravenously with 107 WT or puf2(-) infected erythrocytes. Blood was collected from the mice 4 days later to determine the parasitemia (A), the proportion of gametocytes among parasites (B), and the number of exflagellation centers per μl of blood (C). Results are expressed as mean ± SEM. **, p < 0.01 (Mann-Whitney test).

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Figure 4. Liver infection is severely impaired in puf2(-) parasites. Parasite loads were determined by RT-qPCR analysis of mouse livers (n = 4 or 5 per group) harvested 42 hours after intravenous injection of 10,000 WT, puf1(-) or puf2(-) sporozoites. Results are expressed as the relative expression of Pb18S normalized to mouse GAPDH. The means ± SEM are indicated by lines **, p < 0.01 (Mann-Whitney test).

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sporozoites had transformed, whereas only a minor fraction of WT and puf1(-) sporozoites exhibited signs of premature transformation (Figure 6B). Interestingly, we did not observe expression of the liver stage marker UIS4 or nuclear divisions, as seen in EEFs (Figure 5C), in the transformed puf2(-) sporozoites (Figure 6A).

puf2(-) sporozoites have reduced levels of Puf1 and UIS1/IK2 mRNA
The phenotype of puf2(-) parasites is essentially identical to that of parasites that contain a targeted deletion of the kinase UIS1/IK2 [12]. Similarly to puf2(-) parasites, sk2(-) sporozoites transform prematurely in the mosquito salivary glands and have a decreased infectivity in vivo but not in vitro [12]. Therefore, we sought to test expression of IK2 in puf2(-) sporozoites, in comparison to WT and puf1(-) sporozoites, using RT-qPCR. As expected from gene deletion, no Puf1 and Puf2 mRNA were detected in puf1(-) and puf2(-) sporozoites, respectively (Figure 7). Whereas expression of UIS1/IK2 was not modified in puf1(-) sporozoites, we observed a ~14 fold reduction of UIS1/IK2 mRNA in Puf2-deficient sporozoites as compared to WT (Figure 7). Additionally, we found a ~17 fold reduction of Puf1 transcript levels in puf2(-) parasites. Conversely, Puf2 transcript levels were not affected in the absence of Puf1 (Figure 7). As controls, UIS4 and HSP70 mRNA levels were similar in the mutant and WT sporozoites. Altogether, these data indicate that Puf2 regulates a subset of genes in P. berghei sporozoites, including Puf1 and the kinase UIS1/IK2. The latter probably explains, at least in part, why the phenotype of puf2(-) sporozoites recapitulates that of IK2-knockout parasites.

**Discussion**
*Plasmodium* sporozoites must persist and remain infectious within the salivary glands of the mosquito for many days until they are eventually transmitted to a mammalian host. Inside the warm-blooded host they need to quickly leave the site of deposition in
order to travel to the liver, invade hepatocytes and differentiate into liver stages [29]. The transient developmental arrest of sporozoites inside mosquito salivary glands, termed latency [12], implies efficient control mechanisms to prevent premature transformation before transmission and during transmigration before reaching a suitable host cell. In this study, we identified a factor controlling sporozoite latency in P. berghei, the RNA-binding protein Puf2. In the absence of Puf2, sporozoites transform
prematurely in the mosquito, resulting in a severe loss of infectivity.

Sporozoite conversion into liver stages requires initial remodelling of the parasitic pellicle, with disassembly of the inner membrane complex (IMC) and appearance of a bulb that progressively enlarges until the initially elongated sporozoite has transformed into a round form [28,30]. Previous work has shown that transformation of salivary gland sporozoites is induced at 37°C in culture medium, irrespective of the presence of host cells [28]. It should be noted that differentiation into EEFs involves additional events, including expression of liver stage specific proteins, onset of nuclear divisions and parasite growth. None of these events are observed in axenic culture conditions [28], where instead extracellular sporozoites die rapidly after transformation [31].

We show that puf2(-) sporozoites transform prematurely in the mosquito salivary glands, as evidenced by the characteristic bulb-like structures and rounding-up of the parasites. Premature transformation probably impairs the sporozoite functions that depend on IMC integrity, such as parasite motility, cell traversal and invasion, thus resulting in a loss of infectivity. In the absence of Puf2, the proportion of transformed salivary gland sporozoites increases over time, which correlates with a progressive loss of infectivity to mice. Interestingly, although most puf2(-) sporozoites eventually transform into completely round forms, these forms do not progress to EEF differentiation, as shown by minimal expression of the liver stage marker UIS4 and absence of nuclear division or growth. In contrast, normal differentiation of puf2(-) parasites is observed once sporozoites invade cultured hepatoma cells. Collectively, these data strongly suggest that Puf2 plays a major role in preventing premature remodelling of the sporozoites prior to liver infection, but is not required for EEF differentiation.

The defects observed in puf2(-) parasites are reminiscent of those described in ik2-knockout parasites [12]. Both puf2(-) and ik2(-) sporozoites transform prematurely in the mosquito and display greatly reduced infectivity to mice. However, both loss-of-function mutants are able to invade and differentiate into EEFs in cultured cells in vitro, indicating that they do not play any essential role after host cell infection. These observations, combined with a major down-regulation of IK2 expression in puf2(-) sporozoites, suggest that the phenotype of Puf2-deficient parasites can be explained, to a large extent, by IK2 depletion.

How IK2 prevents sporozoite transformation has yet to be determined. Phosphorylation of the alpha subunit of eIF2 by distinct kinases, such as Plasmodium IK2, is a central mechanism in stress-induced translational regulation [32], including in protozoans. For example, the eIF2alpha kinase IK1 regulates responses to starvation stress in P. falciparum blood stages [13], and in Toxoplasma gondii, phosphorylation of eIF2alpha promotes survival of extracellular tachyzoites [33]. Our data corroborate the findings of Zhang et al. [12], which together suggest that a similar stress response operates in sporozoites to maintain them in a quiescent stage.

The founding member of the Puf family, Drosophila melanogaster Pumilio (DmPUM), regulates, amongst other functions, abdominal development in the fly via translational repression of the maternally inherited hunchback (hb) mRNA [34]. The Puf domain of DmPUM binds to a nanos response element (NRE) sequence located in the 5' UTR of hb mRNA. Biochemical data, such as in vitro binding assays using recombinant Puf domains expressed in bacteria and heterologous in vivo studies using the yeast three-hybrid system, have demonstrated intrinsic binding activity of the P. falciparum PfPuf1 and PfPuf2 to the NRE sequence [20,21]. Signature RNA sequences that are recognized by the Puf domain vary between species and members of the Puf family, but typically contain a UGUR motif [35,36,37,38]. A large number of Plasmodium genes contain UGUR motifs in their 3' UTR, but their functional significance remains uncertain, especially in the context of the exceptional AT-richness of the Plasmodium genome. Therefore, endogenous targets of Plasmodium Puf proteins still remain elusive. The P. falciparum [22] and P. berghei (this study) mutants now constitute potential tools to identify Puf2 target genes in Plasmodium. In sporozoites, Puf2 regulates at least two other genes in addition to IK2 and PfPuf1. Indeed, using RT-qPCR, we found a 4-fold reduction of Spect and Spect2 mRNA levels in puf2(-) sporozoites, whereas actin and AMA1 were not affected (unpublished data). Reduced expression of Spect and Spect2 genes, which are both essential for sporozoite cell traversal and migration to the liver [29,39,40], may also contribute to the loss of infectivity of puf2(-) parasites in vivo.

Whereas Puf proteins typically modulate target mRNA expression by either promoting mRNA turnover or translational repression, they can also activate gene expression or control mRNA subcellular localization (reviewed in [18] and [19]). Our results are not compatible with a role of Puf2 in repressing IK2, because puf2(-) and ik2(-) share a similar phenotype. Puf2 may instead participate in stabilization of IK2 transcripts. Alternatively, depletion of IK2 mRNA in puf2(-) could be an indirect effect due to activation of an upstream factor that regulates IK2.

Whereas DmPUM encodes only DmPUM, many organisms, including C. elegans, contain two or more genes encoding Puf proteins, which can fulfil partly redundant functions [41]. Therefore, presence of two Puf genes in the Plasmodium genome might be explained by overlapping or distinct roles. However, our molecular genetics data clearly exclude a vital role for Puf1 under normal conditions throughout the P. berghei life cycle. Puf1 may be critical under specific conditions, similarly to the role of IK1 in P. falciparum during starvation-induced stress [13]. Puf2 might compensate for the absence of Puf1 in puf1(-) parasites, but not vice versa. While Puf1 in principle might be able to functionally complement for Puf2 function, depletion of Puf1 at the mRNA level precludes a hypothetical functional overlap in vivo. In this regard, it should be noted that P. falciparum
P. berghei Pufl and Pufl2 proteins are very different in size (1183 versus 477 amino acids, respectively), and share only little homology (~27% identity) restricted to the Pufl domains. P. falciparum parasites that lack Pufl2 show increased gametocyte rates and a bias towards male gametocytes [22]. These observations fit with the proposed unifying, and perhaps ancestral, role of Pufs in promoting cell proliferation and repressing differentiation [18]. Our findings that Pufl2 inhibits sporozoite transformation further support the notion of a central role in suppression of cellular differentiation. Because of the published data from P. falciparum pufl2(-) parasites we did not investigate sexual development and differentiation of Pfpufl2(-) parasites in great detail other than to confirm the previous findings, i.e. an increase in gametocytogenesis in pufl2(-) parasites, partly due to increased male gametocyte differentiation. In the previous study, life cycle progression of Pfpufl2(-) parasites beyond gametocytogenesis was not analyzed [22]. Based on our results in the rodent malaria model system, we predict that P. falciparum sporozoites lacking Pufl2 will present a similar phenotype, that is premature sporozoite transformation in the mosquito and decreased infectivity. Therefore, our findings might be of considerable interest in the development and differentiation of P. berghei Puf1(-) parasites beyond gametocytogenesis was analyzed [22]. Based on our results in the rodent malaria model system, we predict that P. falciparum sporozoites lacking Pufl2 will present a similar phenotype, that is premature sporozoite transformation in the mosquito and decreased infectivity. Therefore, our findings might be of considerable interest in the development and differentiation of P. berghei Puf1(-) parasites beyond gametocytogenesis. In the previous study, life cycle progression of Pfpufl2(-) parasites beyond gametocytogenesis was not analyzed [22]. Based on our results in the rodent malaria model system, we predict that P. falciparum sporozoites lacking Pufl2 will present a similar phenotype, that is premature sporozoite transformation in the mosquito and decreased infectivity. Therefore, our findings might be of considerable interest in the development and differentiation of P. berghei Puf1(-) parasites beyond gametocytogenesis was analyzed [22]. Based on our results in the rodent malaria model system, we predict that P. falciparum sporozoites lacking Pufl2 will present a similar phenotype, that is premature sporozoite transformation in the mosquito and decreased infectivity.

Materials and Methods

Ethics statement

All animal work was conducted in accordance with the German ‘Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)’, which implements the directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocol was approved by the ethics committee of MPI-IB and the Berlin state authorities [LAGeSo Reg # G0469/09].

Experimental animals, parasites and cell lines

Female NMRI and C57BL/6 mice were from Charles River Laboratories. We used P. berghei ANKA clone 507 parasites, which constitutively express the green fluorescent protein (GFP) [26]. HepG2 cells (ATCC HB-8065) were cultured as described [43].

P. berghei Pufl and Pufl2 gene deletion

A targeting construct for Pufl gene knockout was generated by inserting a 503-bp 5’ fragment and a 575-bp 3’ fragment on either side of a T. gondii DHFR/TS expression cassette. A construct for Pufl2 gene knockout was generated by inserting a 1001-bp 5’ fragment and a 943-bp 3’ fragment on either side of a human DHFR expression cassette. Oligonucleotide sequences are indicated in Table S1. P. berghei parasites were transfected with linearized plasmids, using the Nucleofector® device (Amaxis GmbH) as described [44], injected intravenously into naive NMRI mice, and selected by pyrimethamine treatment in the drinking water. Clonal parasite populations were obtained by limiting dilution series and intravenous injection of one parasite in 10 recipient NMRI mice. One pufl(+ /- ) and two pufl2(- /- ) clonal parasite lines were established and phenotypically characterized. Genotyping of WT and recombinant parasites was performed by PCR and Southern blot analysis of genomic DNA. Standard Southern blot analysis was performed using the PCR DIG Probe synthesis kit and the DIG Luminescent Detection kit (Roche), according to the manufacturer’s instructions.

Real time quantitative RT-PCR

Parasite total RNA was extracted with the RNeasy kit (Qiagen) and reverse transcribed with the RETROScript kit (Ambion). Real time PCR was performed on cDNA preparations as described [11], using the StepOnePlus™ Real-Time PCR System and Power SYBR® Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s instructions. Expression data were normalized using the constitutively expressed G6P gene.

Immunofluorescence

Parasites were fixed in 4% paraformaldehyde (PFA) and permeabilized with 1% Triton X-100. Immunofluorescence was performed using the PCR DIG Probe synthesis kit and the DIG Luminescent Detection kit (Roche), according to the manufacturer’s instructions. Expression data were normalized using the constitutively expressed G6P gene.

Parasite growth and sexual development

C57BL/6 mice (n = 5) were injected intravenously with 107 infected erythrocytes. Four days later, the parasitemia was determined by microscopic examination of Giemsa-stained blood smears. To analyze exflagellation of male gametocytes, five microliters of tail blood were diluted 1:25 in RPMI 1640 containing 10% FCS and 50 µM xanthurenic acid, and adjusted to pH 8.0. After 12 min incubation at room temperature, exflagellation centers were counted in a Neubauer chamber. Mean parasitemia and gametocyte rates were compared using the Mann-Whitney non-parametric test. After parasite transmission to Anopheles stephensi mosquitoes, the numbers of midgut oocysts and salivary gland sporozoites were determined at day 10–14 and day 18–25, respectively, and compared using the Kruskal-Wallis multiple comparison tests.

Analysis of sporozoite in vivo infectivity

C57BL/6 mice were injected intravenously with 1,000 WT or mutant sporozoites isolated from the salivary glands of infected mosquitoes, or exposed to 10 infected mosquito bites, as indicated. Infection was then monitored daily by examination of Giemsa-stained blood smears. The delay of patency was defined as the time before detection of at least one erythrocytic stage in the smears. For quantification of parasite liver loads by real time RT-PCR, C57BL/6 mice were infected intravenously with 10,000 sporozoites. At 42 hours post-infection, livers were harvested, total RNA was extracted with the RNeasy kit (Qiagen) and cDNA synthesized with the RETROScript kit (Ambion). Real-time PCR was then performed with the StepOnePlus™ Real-Time PCR System and Power SYBR® Green PCR Master Mix (Applied Biosystems), using primers specific for P. berghei 18S rRNA and mouse GAPDH, as described [47]. Liver parasite loads were compared using the Mann-Whitney non-parametric test.

Supporting Information

Table S1 List of oligonucleotides used in this study. (PDF)
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