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A mitogen-activated protein kinase regulates male gametogenesis and transmission of the malaria parasite *Plasmodium berghei*

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Differentiation of malaria parasites into sexual forms (gametocytes) in the vertebrate host and their subsequent development into gametes in the mosquito vector are crucial steps in the completion of the parasite's life cycle and transmission of the disease. The molecular mechanisms that regulate the sexual cycle are poorly understood. Although several signal transduction pathways have been implicated, a clear understanding of the pathways involved has yet to emerge. Here, we show that a *Plasmodium berghei* homologue of *Plasmodium falciparum* mitogen-activated kinase-2 (Pfmap-2), a gametocyte-specific mitogen-activated protein kinase (MAPK), is required for male gamete formation. Parasites lacking Pbmap-2 are competent for gametocytogenesis, but exflagellation of male gametocytes, the process that leads to male gamete formation, is almost entirely abolished in mutant parasites. Consistent with this result, transmission of mutant parasites to mosquitoes is grossly impaired. This finding identifies a crucial role for a MAPK pathway in malaria transmission.

Keywords: MAPK; *Plasmodium*; gametogenesis; exflagellation; transmission

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INTRODUCTION

Malaria is responsible for 500 million clinical cases and up to 3 million deaths every year (www.malaria.org). The most severe form of malaria is caused by the protozoan parasite *Plasmodium falciparum*. The disease is transmitted when an infected female *Anopheles* mosquito injects sporozoites into the vertebrate host. The sporozoites migrate to the liver where they undergo schizogony, generating several thousand merozoites. Thereafter, the merozoites invade red blood cells (RBCs), where they again undergo schizogony, releasing merozoites capable of invading new RBCs. Although most parasites replicate asexually, causing the clinical symptoms of malaria, some withdraw from proliferation and differentiate into male and female gametocytes. Once ingested by a mosquito, gametocytes develop into gametes in the insect's midgut; for the male gametocyte, this involves three rounds of genome replication and marked morphological changes, leading to the production of eight flagellated gametes per gametocyte (a process called 'exflagellation'). Fertilization ensues, leading to the formation of ookinetes, oocysts and sporozoites. Thus, transmission of the disease is critically dependent on gametocytogenesis in the vertebrate host and subsequent development in the mosquito. Interference with these processes would represent useful tools in malaria control, especially with respect to prevention of dissemination of drug resistance.

The differentiation into gametocytes in the vertebrate host is not understood at the molecular level, neither with respect to the signals triggering the process nor with respect to the transduction pathways involved (Dyer & Day, 2000). In contrast, signals and pathways that are implicated in male gametocyte exflagellation have been identified. Signals include a drop in temperature, a rise in pH and exposure to mosquito factors such as xanthurenic acid (Nijhout & Carter, 1978; Sinden, 1983; Billker *et al*, 1998; Garcia *et al*, 1998). There is biochemical evidence that these signals activate cyclic GMP, phosphoinositide and calcium signalling pathways (Kawamoto *et al*, 1990, 1993; Ogwan'g *et al*, 1993; Martin *et al*, 1994). A recently published reverse genetics study showed that a calcium-dependent protein kinase, PbCDPK4, is essential for the formation of the male gamete in *Plasmodium berghei* (Billker *et al*, 2004). However, an integrated picture of the signalling pathways that regulate male gametogenesis has yet to emerge.

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Here, we present our findings on the role of a mitogen-activated protein kinase (MAPK) in male gametogenesis. MAPK pathways have central roles in the adaptive response of eukaryotic cells to a wide range of stimuli. They comprise a three-component module composed of a mitogen-activated protein kinase kinase kinase (MAPKKK), a mitogen-activated protein kinase kinase (MAPKK) and a MAPK, the sequential phosphorylation of which is triggered by activation of the MAPKKK, which can occur through a variety of upstream signalling elements. The activated MAPK phosphorylates a variety of substrates, including cell-cycle control elements and transcription factors, and thereby has a central role in cell-cycle arrest and transcriptional reprogramming that accompanies cell differentiation. Recently, instances of MAPKK-independent MAPK activation have been reported (for reviews, see Widmann *et al*, 1999; Raman & Cobb, 2003). Two *P. falciparum* MAPK homologues have been characterized: Pfmap-1 (Plasmo DB gene identifier PF14_0294), which clusters with the extracellular signal-regulated kinase (ERK) 7/8 subfamily of MAPKs in phylogenetic analyses (Ward *et al*, 2004), shows the classical Thr-X-Tyr MAPK activation motif and is expressed in both asexual parasites and gametocytes (Doerig *et al*, 1996; Lin *et al*, 1996; Graeser *et al*, 1997); and Pfmap-2 (PlasmoDB identifier PF11_0147), the expression of which is detectable in gametocytes but not in asexual parasites (Dorin *et al*, 1999). Pfmap-2 presents atypical features relative to mammalian MAPKs: the canonical activation site Thr-X-Tyr is substituted in Pfmap-2 by Thr-Ser-His, and insertions are located in the immediate vicinity of this site, suggesting a divergent mode of activation compared with that of mammalian MAPKs (Dorin *et al*, 1999).

Because of its stage-specific pattern of expression, and considering the role of MAPKs in the control of many developmental events in other systems (Bardwell *et al*, 1994), we suggested that Pfmap-2 might be involved in the regulation of gametogenesis. The rodent malaria parasite *P. berghei* is more amenable than *P. falciparum* to genetic modifications (Thathy & Menard, 2002). To understand the function of map-2, we undertook a gene knockout approach in *P. berghei*.

RESULTS

We identified the sequence encoding Pbmap-2, the *P. berghei* orthologue of Pfmap-2, in the PlasmoDB database (http://www.plasmoDB.org). Pbmap-2 (PlasmoDB identifier Pb_5294-2-125-1861) is 72% identical to Pfmap-2 at the amino-acid level of the full-length predicted protein, and 93% of the catalytic domain (see supplementary Fig 1 online for an alignment). The two protein kinase signature motifs (Bairoch & Claverie, 1988) are present, and the atypical features of Pfmap-2, including the substitution of the Thr-X-Tyr activation site by Thr-Ser-His are conserved in Pbmap-2. Reverse transcription–PCR (RT–PCR) experiments using *P. berghei* showed that, like Pfmap-2, Pbmap-2 is expressed in gametocytes but not in asexual parasites (Fig 1).

To disrupt the *pbmap-2* gene, we constructed an insertion plasmid containing a 661-base-pair (bp) fragment of the gene (Fig 2A). This region contains the putative ATP-binding domain but not the catalytic site, both of which are essential for the kinase to be active. This fragment was placed upstream of a selection cassette expressing a pyrimethamine-resistant human dihydrofolate reductase (hDHFR) enzyme in the pDb.Dh.^Db vector (de Koning-Ward *et al*, 2000). The plasmid was linearized at an engineered *Pst*I site to facilitate recombination (Thathy & Menard, 2002). PCR and Southern blot analyses of DNA from transformed parasites showed that the plasmid was able to integrate into the *pbmap-2* locus, suggesting that Pbmap-2 is not essential for asexual multiplication of the parasite. We then obtained six clones by limiting dilution in mice, of which four were found by PCR to have a disrupted *pbmap-2* locus (Fig 2B). The disruption was confirmed in two clones by Southern analysis (Fig 2C). Full-length *pbmap-2* transcripts were not detectable by RT–PCR in these clones (Fig 2D). A phenotypic analysis was conducted using these two mutant clones (b1,c2), as well as clone a1 obtained in this experiment, which had retained a wild-type *pbmap-2* locus, and the original wild-type parasite, NK65.

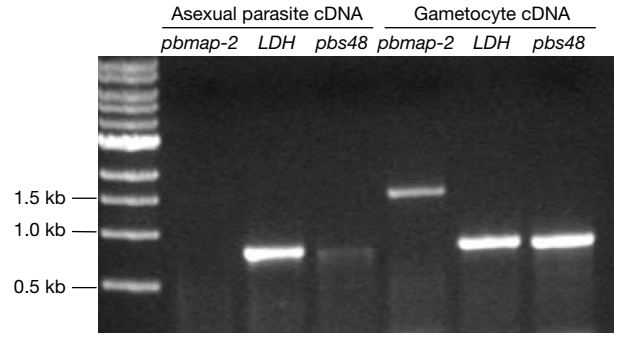

Fig 1 | Expression profile of *pbmap-2*: RT–PCR was performed with RNA from mixed asexual parasites and gametocytes to amplify *pbmap-2*, lactate dehydrogenase (*LDH*) and *pbs48*. *pbmap-2* can be amplified robustly from gametocyte complementary DNA but not from asexual-stage cDNA. *LDH* is expressed in the asexual and sexual stages of the parasite and amplifies readily from both cDNA pools. The gametocyte-specific gene *pbs48* gives a strong signal from gametocyte cDNA and a considerably weaker signal from the asexual parasite cDNA sample (possibly because of contamination of asexual parasite preparation by small numbers of gametocytes).

The phenotypic analysis examined whether Pbmap-2 has any role in (i) gametocytogenesis, (ii) male gametogenesis and (iii) infectivity to the mosquito vector. To address the issue of gametocyte formation, we induced synchronized infections in rats and determined the number of gametocytes per 10,000 RBCs. We found that gametocytes were produced in all three clones with a frequency that was similar to that shown by wild-type parasites. The conversion rate was 3.9% in NK65 parasites, 8.6% in the a1 clone containing a wild-type Pbmap-2 locus, 5.2% in the knockout b1 clone and 9.6% in the knockout c2 clone (see supplementary Table 1 online for details). Hence, gametocyte formation *per se* is not impaired in the mutant clones. Further, the ratio of male to female gametocytes in mutant and wild-type parasites was comparable (supplementary Table 1 online). Next, we examined the ability of male gametocytes to undergo exflagellation. In clone a1 with an undisrupted Pbmap-2 locus, the percentage of male gametocytes capable of exflagellation was 39.9%, and in the wild-type parental NK65 strain, it was 59.5%. In sharp contrast, in the knockout c1 clone, we observed no exflagellation, whereas in clone b1, the percentage of exflagellation was 0.3% (Table 1 and Fig 3). This shows that Pbmap-2 is crucial for male gamete formation.

We next assessed the ability of parasites with a disrupted Pbmap-2 to infect the mosquito vector. Female *Anopheles stephensi* mosquitoes were fed on mice infected with the two mutant clones, the wild-type clone and the wild-type parasite. On day 9 post-feeding, mosquitoes were dissected and their midguts were examined for oocysts. It was found that 73% and 100% of mosquitoes fed on NK65 and clone a1 parasites, respectively, were positive (see supplementary Table 2 online for details). In contrast, we did not observe any oocysts in mosquitoes fed on mutant clones b1 and c2. We, however, observed a small number of sporozoites (16 sporozoites per mosquito) in mosquitoes fed on clone b1, which showed residual exflagellation capability (see Fig 3), and no sporozoites were observed in mosquitoes fed on clone c2. Sporozoite numbers observed in mosquitoes fed on clone a1 or NK65 parasites were 6,000 and 5,666 per mosquito, respectively.

DISCUSSION

Our results show that an intact *pbmap-2* gene is crucial for exflagellation *in vitro* and for successful completion of the sexual cycle in the mosquito.

Because no additional selectable markers are available for use in *P. berghei* clones that possess the hDHFR selection cassette, we

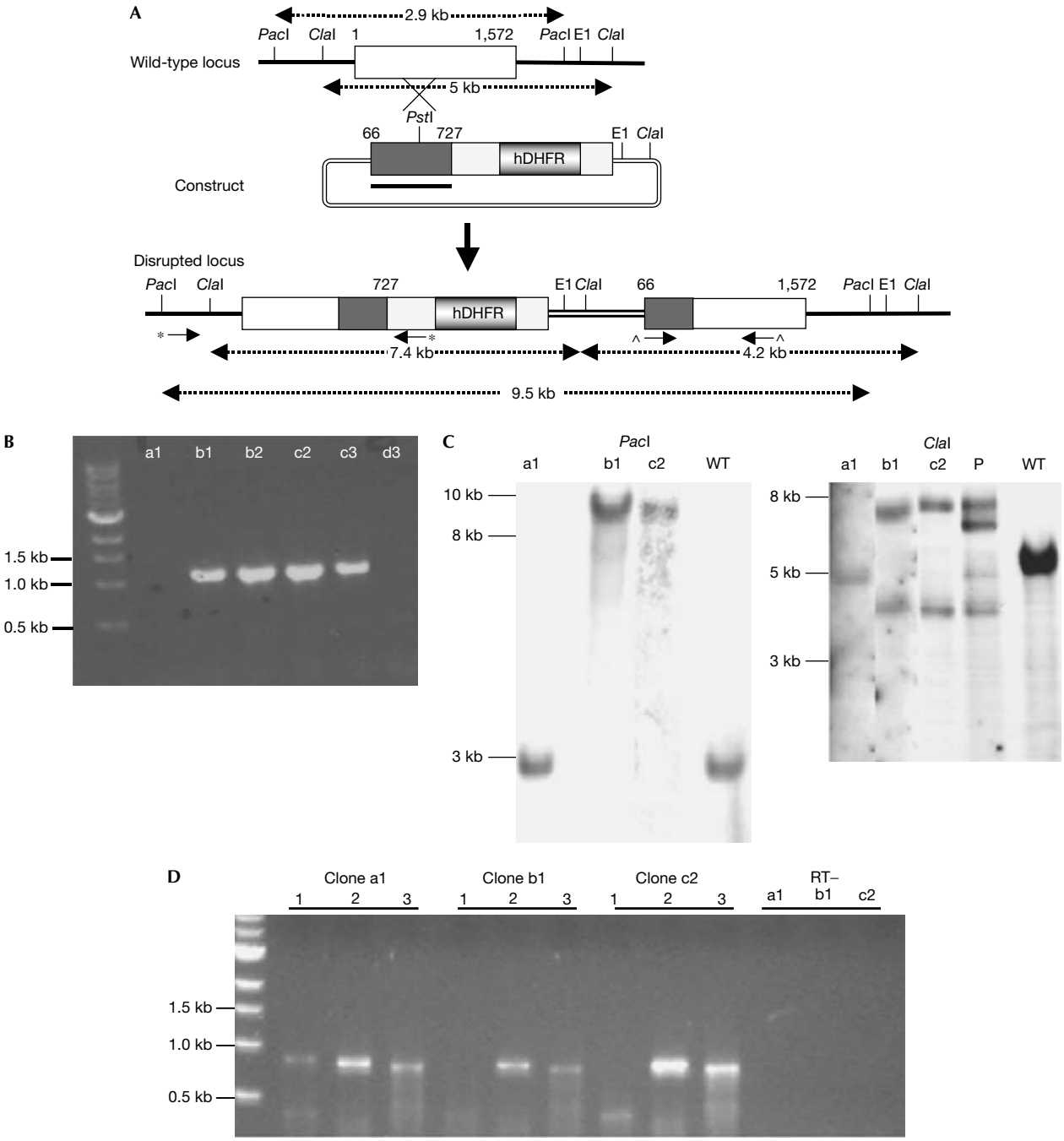

Table 1 | Exflagellation rates in wild-type parasites and *pbmap-2* knockout mutants

Genotype	Exflagellations per 10,000 RBCs	Male gametocytes per 10,000 RBCs	Percentage of exflagellation
NK65	8.8	14.8	59.5
a1 (WT)	5.5	13.8	39.9
b1 (KO)	0.05	16.2	0.3
c2 (KO)	0	11.8	0

KO, knockout; RBC, red blood cells; WT, wild type; see the Methods for details.

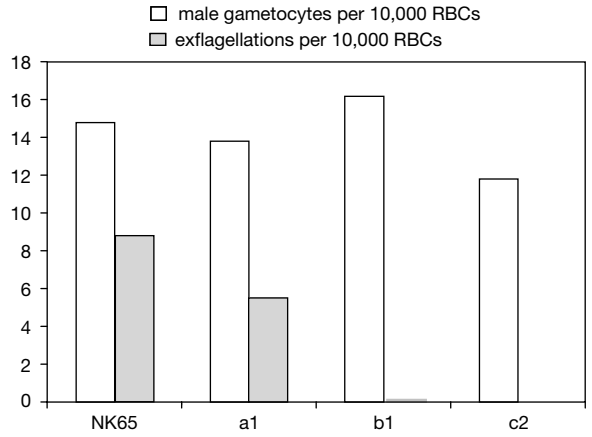

Fig 3 | Number of male gametocytes (white bars) and exflagellation centres (grey bars) per 10,000 RBCs counted in triplicate in two animals per clone. Details of data are presented in Table 1.

lack the tools to perform a *pbmap-2* knock-in experiment to verify that the wild-type phenotype of normal exflagellation rate can be restored. However, the fact that mutant parasites are able to undergo not only the entire asexual cycle, but also differentiation into gametocytes, argues strongly against the possibility that the phenotype results from a nonspecific or pleiotropic effect. Furthermore, the two independent clones b1 and b2 show the phenotype, in contrast to the a1 control clone that has undergone the transfection/selection procedures but retained an undisrupted *pbmap-2* locus. Taken together, these observations provide strong evidence that the effect is because of disruption of the Pbmap-2 locus.

What role might Pbmap-2 have in regulating exflagellation? Although the loss of Pbmap-2 does not prevent differentiation into male gametocytes, these mutant gametocytes may be defective in DNA synthesis, mitosis or motility. Several studies have shown that exflagellation in *P. berghei* and *P. falciparum* is sensitive to inhibitors that block the release of intracellular calcium and to calmodulin antagonists (Kawamoto *et al*, 1990). Physiological amounts of xanthurenic acid induce a rapid increase of intracellular calcium in the *P. berghei* gametocyte, and the calcium-dependent protein kinase CDPK4 was identified as a regulator of exflagellation (Billker *et al*, 2004). In addition to calcium release, xanthurenic acid treatment is likely to cause the previously observed increase in intracellular cGMP during exflagellation (Kawamoto *et al*, 1993), as a xanthurenic acid-dependent guanylyl cyclase activity has been detected in gametocyte membranes (Muhia *et al*, 2001). This is likely to stimulate protein kinase G (PKG), a homologue of which has been characterized in *P. falciparum* (Deng & Baker, 2002). The identification of Pbmap-2 as another protein kinase controlling exflagellation raises the question of the interplay of the signal transduction pathways in which PfCDPK4, Pfmap-2 and presumably PfPKG operate to coordinate male gametogenesis.

The small number of exflagellating gametocytes and sporozoites produced by clone b1 may be because of low levels of *pbmap-2* expression in these parasites (below the detection limit of our RT–PCR experiments) generated from the copy containing residues 66–1,572, despite the absence of a bona fide promoter. Another possible explanation for the appearance of small numbers of sporozoites in clone b1 might be inefficient complementation by another kinase. Pfmap-1, expressed in *P. falciparum* asexual parasites and gametocytes, has an orthologue in *P. berghei*, which may be a candidate for a putative Pbmap-2 complementation activity.

Malaria parasites do not possess typical MAPKKs, as illustrated by repeated failures to identify such homologues by database mining, homology-based gene amplification and analysis of the *P. falciparum* kinome (Ward *et al*, 2004; Dorin *et al*, 2005). Hence, it is likely that Pb/Pfmap-2 activation is not achieved in the

◀ **Fig 2** | Strategy for inactivation of the *pbmap-2* gene by single cross-over homologous recombination, and genotypic characterization of mutant parasites. (**A**) Schematic of the wild-type locus, the targeting construct and the disrupted locus. The targeting construct spans positions 66–727 of the 1,572 bp coding sequence (see arrows in Fig 1 of supplementary information online). The disrupted locus contains two nonfunctional copies of the gene. The first copy is truncated at position 727 and lacks essential catalytic residues, whereas the second copy is unlikely to be expressed as it lacks 5′ promoter sequences and the first 65 residues of the coding sequence. (**B**) Clones recovered by limiting dilution were tested for disruption of the locus by PCR using primers indicated by inverted arrows marked with an asterisk in (**A**) (primers 1,2; see supplementary Table 3 online for a list of primer sequences). We were able to amplify a fragment of the expected size (1,153 bp) in four of the six clones (b1,b2,c2,c3), suggesting that these clones had undergone disruption of the *pbmap-2* locus. (**C**) The parental population P (which was used for the limiting dilution), clone a1 (with a wild-type Pbmap-2 locus) and the knockout clones b1 and c2 were selected for Southern blot analysis. DNA was digested with *Pac*I or *Cla*I and probed with a fragment (66–727 bp) underlined in black in the targeting construct. The expected sizes of the restriction fragments are indicated in (**A**). The *Cla*I blot for the parental population P had bands representing the episomal (6.6 kb), wild-type and disrupted *pbmap-2* loci. Both blots showed that clones b1 and c2 had a disrupted *pbmap-2* locus. (**D**) RT–PCR with 1–1.5 μg of RNA from a1, b1 and c2 clones was performed with primers marked with a '^' in (**A**); primers 3,4 in supplementary Table 4 online). The region between the primers can be amplified only if full-length *pbmap-2* is transcribed. In clone a1, we observed an amplification product of the expected size (869 bp) but not in clone b1 or c2 (lane 1 for each clone). We amplified *LDH* (primers 5,6) and *pbs48* (primers 7,8; lanes 2 and 3, respectively, for each clone) as controls. No products were amplified from *LDH* reactions without reverse transcriptase (RT−).

context of a typical three-component MAPK module (MEKK–MEK–MAPK (MEK: MAPK kinase)) as found for ERK1/2 in other eukaryotes. However, incubation of Pfmap-2 in extracts from gametocytes (but not from asexual parasites) stimulated its activity, indicating that this enzyme operates in a transduction pathway (Dorin *et al*, 1999). Interestingly, a study conducted *in vitro* showed that Pfnek-1, a kinase related to the NIMA/Nek family (Nek stands for NIMA-related kinase, where NIMA (never in mitosis/Aspergillus) is a protein kinase required for G2-M transition in Aspergillus), was able to specifically phosphorylate Pfmap-2, but neither Pfmap-1 nor typical MAPKs such as mammalian ERK2. Pfnek-1 and Pfmap-2 acted synergistically to phosphorylate an exogenous substrate, suggesting that phosphorylation of the MAPK by Pfnek-1 may result in its activation (Dorin *et al*, 2001). Furthermore, mutagenesis studies have shown that the Thr and the His residues (but not the Ser residue) in the Thr-Ser-His putative activation motif of Pfmap-2 are required for activity of the recombinant enzyme (Dorin *et al*, 1999). These data point to a MAPK activation pathway that is unique to *Plasmodium*.

Further studies aimed at dissecting the pathways leading to exflagellation, and in particular at identifying protein substrates for Pb/Pfmap-2, Pb/PfCDPK4 and Pb/PfPKG, are necessary to explain the specific mechanisms pertaining to *Plasmodium* gametogenesis. Such studies constitute the basis for the rational development of transmission-blocking drugs, which would represent an invaluable addition to malarial control strategies. Indeed, the four protein kinases mentioned above are active *in vitro* as recombinant enzymes (PfPKG: Deng & Baker, 2002; Pfmap-2: Dorin *et al*, 1999; Pfnek-1: Dorin *et al*, 2001; PfCDPK4: O. Billker, D.D. and C.D., unpublished), and some of these are being used at present in the screening of chemical libraries as a first step towards drug discovery. Reverse genetics studies aimed at defining the role of these enzymes in the parasite's life cycle, such as the Pbmap-2 knockout analysis presented here, are essential in terms of target validation.

METHODS

Expression analysis. Asexual parasites and gametocytes were isolated from rats infected with NK65 parasites (see http://www.azl.nl/1040/research/malaria/model.html for protocols). RNA was extracted using the RNAeasy kit (Qiagen, Hilden, Germany). A 1–1.5 μg portion of RNA was reverse transcribed using the 3′ RACE kit (Invitrogen, Carlsbad, CA, USA, cat. no. 18373-079).

Genotype analysis. Parasite DNA was isolated (Thathy & Menard, 2002) and subjected to PCR (primers 1,2; see supplementary Table 3 online) and Southern analysis. Parasite DNA was digested with *Pac*I or *Cla*I and transferred to a nylon membrane. The membrane was hybridized with a digoxigenin (DIG)-labelled probe diluted in DIG easy Hyb (Roche, Indianapolis, IN, USA) overnight at 37 °C. The membrane was incubated with anti-DIG antibodies conjugated to alkaline phosphatase (1:10,000) for 30 min at 25 °C. A chemiluminescent substrate, CSPD (a chloro substituted 1,2-dioxetane; Roche), was added to the membrane. The membrane was exposed to a film for 5–15 min.

Phenotype analysis. Gametocytogenesis: Synchronized infections were induced in rats and the number of ring- stage parasites was determined at 24 hours post infection (hpi) in blood smears. At this time, asexual parasites and nascent gametocytes are indistinguishable. Therefore, the parasites were allowed to grow for another 6 h. Fully differentiated gametocytes were counted at 30 hpi and expressed as a percentage of the total number of parasites counted at 24 hpi (conversion rate). Around 10,000 RBCs were counted per animal per time point. Male and female gametocytes were distinguished microscopically in Giemsa-stained blood smears by size and coloration. Exflagellation assay: Blood (2–3 μl) was collected from the tail of infected mice and mixed with 15 μl of RPMI 1640 with 25 mM HEPES and 10% fetal calf serum (pH 8.2). A 10 μl portion of this blood-medium mix was put on a slide, covered, sealed and left at 25 °C for 15 min. The number of exflagellation centres was counted for 10 min (around 30,000 RBCs). Finally, a tail smear was made from the same animal and the number of male gametocytes was determined per 10,000 RBCs. The data were used to calculate the percentage of male gametocytes capable of exflagellation. Counts were made in triplicate in two infected mice per clone.

Transmission. A total of 75–100 female *A. stephensi* mosquitoes were fed on one or two infected mice. On day 10 post-feeding, five mosquitoes were dissected and examined for oocysts. Percentage infectivity (the number of mosquitoes positive for oocysts) was calculated. On day 19, salivary glands from five to ten mosquitoes were dissected, gently crushed and the sporozoites were pooled. The number of sporozoites was counted using a haemocytometer and used to estimate the number of sporozoites per mosquito. The data are an average of three separate experiments, except for the wild-type clone a1 for which data are an average of two experiments.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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