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A putative kinase related protein (PKRP) from *Plasmodium berghei* mediates infection in the midgut and salivary glands of the mosquito

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Abstract

The completion of the *Plasmodium* (malaria) life cycle in the mosquito requires the parasite to traverse first the midgut and later the salivary gland epithelium. We have identified a putative kinase related protein (PKRP) that is predicted to be an atypical protein kinase, which is conserved across many species of *Plasmodium*. The *pkrp* gene encodes a RNA of about 5,300 nucleotides that is expressed as a 90 kDa protein in sporozoites. Targeted disruption of the *pkrp* gene in *Plasmodium berghei*, a rodent model of malaria, compromises the ability of parasites to infect different tissues within the mosquito host. Early infection of mosquito midgut is reduced by 58-71%, midgut oocyst production is reduced by 50-90% and those sporozoites that are produced are defective in their ability to invade mosquito salivary glands. Midgut sporozoites are not morphologically different from wild type parasites by electron microscopy. Some sporozoites that emerged from oocysts were attached to the salivary glands but most were found circulating in the mosquito hemocoel. Our findings indicate that a signaling pathway involving PpPKRP regulates the level of *Plasmodium* infection in the mosquito midgut and salivary glands.

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Note: Supplementary data associated with this article.

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Keywords

Malaria; *Plasmodium berghei*; Transmission; Ookinete; Oocyst; Sporozoites; Invasion; Epithelium; Midgut; Salivary gland; PKRP; Kinase

1. Introduction

The transmission of malaria parasites from the bites of *Anopheles* mosquitoes is responsible for the millions of clinical cases and deaths from malaria each year (Mayor, 2008). The complex life cycle of the parasite requires an intricate network of signaling events to enable the parasite to invade multiple cell types in both the invertebrate and mammalian hosts (Ward et al., 2004; Doerig et al., 2005). Two invasive life cycle stages that occur exclusively in the mosquito, the ookinete and the sporozoite, require invasion into the mosquito midgut and salivary gland epithelium, respectively. Ookinetes cross the midgut epithelium within 24 h of a blood meal and produce oocysts. Sporozoites are released from the oocysts into the hemocoel about 18 days later and invade the salivary glands by passing through the epithelium, completing the development of the parasite in the mosquito.

The mechanism used by the parasite to invade mosquito epithelial tissues is unknown but is thought to involve a signaling pathway. The parasite needs to transverse two epithelia in the mosquito, first the midgut and later the salivary gland, to complete the infection cycle. A peptide (SM1) that binds specifically to these two epithelia and inhibits the attachment of the parasite has been identified (Ghosh et al., 2001). Transgenic mosquitoes that over-expressed this peptide in the midgut epithelium were less susceptible to *Plasmodium berghei* infection, and contained fewer sporozoites in the salivary glands (Ito et al., 2002). These interactions between parasite proteins and receptors on the mosquito epithelium may initiate a downstream signaling cascade to facilitate parasite invasion.

Protein kinases, such as calmodulin-dependent protein kinases (CaMKs), are involved in many cellular processes, including invasion of cells (Bonhomme et al., 1999; Griffith, 2004). In *Plasmodium*, the CaMK family includes calcium-dependent protein kinases (CDPKs), as well as a distinct group that does not contain EF-hand motifs (important for calcium-binding) (Ward et al., 2004). One calcium-dependent protein kinase, CDPK3, is required for ookinete motility in *P. berghei*, which in turn is required for efficient mosquito midgut invasion (Ishino et al., 2006; Siden-Kiamos et al., 2006). CDPK4 is essential for the sexual reproduction and mosquito transmission of *P. berghei*, as it is involved in the regulation of cell cycle progression in the male gametocyte (Billker et al., 2004). Another member of this family, CDPK6, is involved in signaling, mediating the activation of sporozoites for invasion (Coppi et al., 2007). Other parasites, including *Toxoplasma gondii* and *Cryptosporidium parvum*, also utilize CDPK-like proteins in invasive stages (Bonhomme et al., 1999; Nagamune and Sibley, 2006). This suggests that the CaMK family may play an important role in cell invasion in many parasite species.

Here, using a *P. berghei* knock-out mutant of a putative kinase-related protein (PKRP), we show that PKRP is involved in mediating infection in the midgut and salivary glands of the mosquito. Ookinetes deficient in this protein do not efficiently infect the midgut epithelium, resulting in a reduction in the number of midgut sporozoites. Most sporozoites that are released from oocysts fail to invade salivary gland epithelium and are predominantly found circulating in mosquito hemocoel. We suggest that the *P. berghei* PKRP (PbPKRP) may be involved in a signaling pathway that mediates the parasite's ability to invade mosquito tissues.

2. Materials and methods

2.1. Multiple sequence alignment

The orthologs of the various *Plasmodium* *pkrp* genes were predicted using OrthoMCL (Li et al., 2003). Multiple sequence alignments of the first 500 amino acids of *Plasmodium falciparum* PKRP (PfPKRP), PbPKRP, *Plasmodium chabaudi* PKRP (PcPKRP) and *Plasmodium yoelii* PKRP (PyPKRP) were performed using Clustal W (Thompson et al., 1994) on the San Diego Supercomputer Center Biology Workbench server (<http://workbench.sdsc.edu>) and a consensus sequence was determined. To construct the predicted catalytic domain alignment of PfPKRP and PbPKRP, an amino acid BLAST search of both domains was conducted to first retrieve those kinases that showed homology. The human testis-specific serine/threonine kinase (hTSSK) was selected as the most similar protein using OrthoMCL and a multiple sequence alignment was constructed using Clustal W.

2.2. Parasite maintenance and transmission to mosquitoes

All procedures for animal experiments were approved by the New York University School of Medicine Institutional Care and Use Committee. *Plasmodium berghei* ANKA wild-type (wt; clone C₁) and transgenic lines were maintained in Swiss-Webster mice (approximately 4 weeks old; National Institutes of Health). *Anopheles stephensi* mosquitoes were maintained at 70% humidity and 22°C and infected with parasites essentially as previously described (Vanderberg, 1980). Groups of five to eight mice were infected with wt or transgenic lines by i.v. infection and monitored for parasitemia and gametocytemia. Three to five mice from each group with matched parasitemia (differences smaller than 1% between all animals) and gametocytemia (differences smaller than 0.01% between all animals) were chosen to feed mosquitoes. The ranges of parasitemia and gametocytemia between the different experiments were 2-5% and 0.02- 0.05%, respectively.

Infected midguts and/or salivary glands were dissected on ice (Purcell et al., 2008). Hemocoel was dissected using volume displacement (perfusion) essentially as previously described (Hillyer et al., 2007). Whole, dissected salivary glands were treated with trypsin to remove any sporozoites that did not invade the salivary gland as previously described (Sultan et al., 1997). The number of sporozoites for a given experiment was determined using an appropriate dilution of sporozoites in DMEM and counting on a hemocytometer. Ookinete-infected midguts were dissected by removing the midguts from mosquitoes fed 24 h earlier on infected blood. The blood meal was removed and the midgut was washed extensively with PBS.

2.3. Deletion of the *pkrp* gene and genotype analysis

A targeting vector for *Pbpkrp* was constructed in plasmid pL0001 (plasmid b3; b3D.DT^H.D), in which polylinker sites flank a *T. gondii* *dhfr/ts* expression cassette transferring resistance to pyrimethamine. A 457 bp fragment of the 5' untranslated region (UTR) sequence was PCR amplified from *P. berghei* genomic (g)DNA using the primers G1FKp (5'-agaaggttaaccacatcataataatagcaactgcac - 3'; restriction site underlined) and G1RH3 (ttagaagctttccagacgggtaatatcaaaa - 3'). The fragment was inserted into the *KpnI* and *HindIII* restriction sites upstream of the *dhfr/ts* cassette of pL0001. A 800 bp fragment of the 3' UTR sequence was PCR amplified from the same gDNA using the primers G2FBam (ttatggatcctgaaatagataaaaattcaatcatgg - 3'; restriction site underlined) and G2RXb (aaaatctagattttcacct - 3'). The fragment was inserted into the *BamHI* and *XbaI* restriction sites downstream of the *dhfr/ts* cassette of the vector. The replacement construct was excised as a *KpnI/SacII* fragment and used for the electroporation of cultured *P. berghei* ANKA wt (clone C₁) schizonts essentially as previously described (Janse et al., 2006). *Plasmodium berghei* ANKA was previously cloned to generate a homogenous wt parasite population. This cloned wt was used to perform two independent transfections where *Pbpkrp* clone 1 was generated

independently of clone 2. Two independent rounds of limiting dilution cloning of drug-resistant parasites were completed, and genotyping of Clone 1 (L₁₀) and Clone 2 (O₁) was carried out. Diagnostic PCR was used to amplify a 250 bp fragment of the *pkrp* gene using the primers ORF F (5' – ccttttgctatgcatggtgat – 3') and ORF R (5' – cattaaatggagggttc – 3'). To confirm 5' integration, a 837 bp fragment of the 5' region was amplified using two primers: the forward primer (Pb837; 5' – caacaaccaattgcatggac – 3') amplified a region upstream of the flanking sequence used to integrate the plasmid along with a reverse primer (Pb103; 5' – taattatgtttattttccac – 3') located 3' of the flanking sequence, but 5' to the *dhfr/ts* sequence. To confirm 3' integration, a 1,549 bp fragment of the 3' region was amplified using two primers: the forward primer (Pb106a; 5' – tgatgcacatgcatgtaaatagc – 3') amplified a region located 3' of the *dhfr/ts* sequence along with a reverse primer (Pb1549; 5' – cattgtatgccgtttggttc – 3') that amplified the region downstream of the flanking sequence used to integrate the plasmid. A negative control of *P. berghei* gDNA alone was also analysed with each PCR reaction. Southern blot analysis was carried out as per the manufacturer's instructions (Roche). A labeled probe was PCR-amplified using the ORF F/ORF R primer set described above. Northern blot analysis on a standard formaldehyde gel was completed using the DIG kit (Roche) by analyzing 5 µg of total RNA extracted from sporozoites of wt or the transfected clones. A sample of the same labeled probe from the Southern blot was used to label the northern blot.

2.4. Antibody production and western blot analysis

A synthetic peptide (N - LFENEKNGLIYPVLNDPGQAIYF - C; Biosynthesis Inc., Lewisville, TX, USA) derived from the predicted catalytic region of PbPKRP (residues 301 to 324) was used for immunization of rabbits (Cocalico Biologicals Inc., Reamstown, PA, USA; see Fig. 1A). For western blotting, 7.5×10^4 sporozoites were dissected from each clone and separated on 8% SDS-PAGE gel and on 4-20% Novex Tris-Glycine gradient gels (Invitrogen). Rabbit antisera (from naïve and immunized rabbits) were used at a 1:300 dilution for western blotting using the ECL western blotting kit (Amersham).

2.5. Extraction of gDNA from mosquito midguts and salivary glands

Midguts (ookinete or oocyst stage) and salivary glands ($n = 10$) from each clone were dissected from mosquitoes as described above and placed in 500 µl oocyst lysis buffer (100 mM NaCl, 25 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.8), 0.5% Sarkosyl and 1 mg/ml proteinase K). The mixture was incubated overnight in a 56°C water bath. Genomic DNA was then isolated using a phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 100 µl of 10 mM Tris, 1mM ethylenediaminetetraacetic acid buffer.

2.6. Quantitative PCR

Genomic DNA isolated from each clone was diluted equally to the indicated concentrations before running the assay. Quantitative PCR reactions were performed in triplicate as previously described (Yanow et al., 2006). For midgut amplifications, DNA was diluted 1:4 and for salivary gland reactions, the DNA was diluted 1:14. For each 25 µl reaction, 5 µl of the diluted gDNA were added. The following oligonucleotides were used: *P. berghei* 18S rRNA gene: 5' – ggcaacaacaggtctgtg-3' and 5' – gtacaaaggcaggacg-3'; *P. berghei* *ama-1* gene: 5' – accggtgatcagtcagtgaagaagt-3' and 5' – gctacaatatcttgaccc-3'; *Pbpkrp*: 5' – ccttttgctatgcatggtgat – 3' and 5' – cattaaatggagggttc – 3'. The percentage of amplification efficiency (AE) was calculated using the formula $\% AE = 2^{\Delta C_t} * 100$, where C_t is the cycle threshold, as previously described (Sikorsky et al., 2004; Yanow et al., 2008), except the % AE was calculated using the wt as unmodified and *pkrp* clone as modified templates.

2.7. Electron microscopy

Infected mosquito midguts were dissected at day 10 p.f. and fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS for 5 days. Specimens were post-fixed in 1% osmium tetroxide and 1.5% $K_3Fe(CN)_6$ in PBS for 2 h at room temperature (RT), followed by incubation in 0.5% uranyl acetate for 1 h. Midguts were dehydrated in increasing concentrations of ethanol and then incubated for 1 h in propylene oxide, followed by another incubation for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences, Hatfield, PA, USA). Specimens were subsequently embedded in Epon at 60°C for 2 days. Post-staining of sections was done with 1% uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope and scanned images were processed using Adobe Photoshop CS software.

2.8. Statistical analyses

All statistical analyses were completed using Prism (v. 4.0a). Where the Area Under the Curve (AUC) was calculated, the average AUC was calculated for each cycle, using software default parameters, and then the average of all AUCs was calculated. Where differences in AUC and quantitative PCR were assessed, normality was tested using the Kolmogorov-Smirnov Goodness-of-Fit Test. Data with a *P* value > 0.10 were considered normal. The differences were then tested using an ANOVA with a Tukey's Multiple Comparison post-hoc test, except for the assessment of oocysts in Fig. 4B, where a two-way ANOVA was used with a Bonferroni post-hoc test. Assays showing significant differences (*P*<0.05) were noted.

3. Results

3.1. Bioinformatic analysis of the PKRP

We bioinformatically analysed a conserved, hypothetical *Plasmodium* protein, and annotated it as a PKRP based on its signature motifs as described below. PfPKRP (*Pfprp*; PFC0485w) is located on chromosome 3. Expression data from this gene shows intermediate level expression in sporozoites, low-level expression throughout the asexual stages and highest levels in gametocytes (PlasmoDB, release 5.3 and Le Roch et al., 2003). The first 500 amino acids containing the N terminus and predicted kinase domain of the various *Plasmodium* PKRP proteins show high homology between species: PbPKRP (PB001650.02.0) is 93% identical to the counterpart in PyPKRP (PY02490), while PfPKRP is 57% and 60% identical to PbPKRP and PyPKRP, respectively (Fig. 1A). All of these *Plasmodium* genes contain two exons, with the predicted protein kinase region in the first exon. The predicted size of the proteins vary with PfPKRP, PbPKRP, PyPKRP and PcPKRP (*Pcprp*; PCAS_041030) comprising proteins of 2,515, 2,708, 2,699 and 2,456 amino acids, respectively. The PbPKRP sequence is not complete in the current available version of PlasmoDB, however the complete predicted sequence was provided from the working copy of the assembly of *P. berghei* ANKA by the Pathogen Genomics group at the Wellcome Trust Sanger Institute, Cambridgeshire UK (Supplementary Fig. S1).

Various searches were carried out to identify a human orthologue of the *Plasmodium* PKRP. None of the full-length PKRP sequences were clear orthologs of other Ser/Thr protein kinases, but kinases sharing sequence identity with the predicted catalytic domain of PKRP were predicted. Several residues that are largely conserved in Ser/Thr protein kinases were identified in the PKRP sequences. One of these kinases, the human testis-specific serine kinase (hTSSK; Hao et al., 2004) has been described as a CaMK-related protein kinase that is classified as atypical, as it contains only a single glycine in the glycine loop motif. TSSK1 shares limited identity with PKRP. The catalytic domains of hTSSK (261 amino acids) shares 26% identity with PfPKRP and 25% identity with PbPKRP (281 amino acids) (Fig. 1B). The catalytic domains of PfPKRP and PbPKRP share 75% identity.

3.2. Generation of parasites lacking PKRP

To characterize the function of PKRP, we performed targeted gene deletion in *P. berghei* (Waters et al., 1997), a parasite species that can infect mice and be transmitted to mosquitoes, which is an established model for malaria transmission. To disrupt the *Pbprkp* gene, the 503 amino acids predicted to compose the full-length protein were replaced with a pyrimethamine-resistance allele of the *dhfr/ts* M2/M3 gene from *T. gondii* (Fig. 2A). Following drug selection, the targeting construct was shown to have integrated into the genome of the parasite. This was confirmed in two clones, designated clone 1 (Fig. 2B and C) and clone 2 (Fig. 2C), isolated independently from the transfected population, using PCR and Southern blot analysis.

The predicted sequence of *Pbprkp* is 8,127 bp after splicing. Northern blot analysis of mixed blood stage RNA isolated from wt parasites and both *Pbprkp*⁻ clones revealed a broad area of hybridization with a major RNA band at approximately 5,300 bp in wt RNA that was absent from the knock-out clones (Fig. 3A and B). Protein was extracted from the sporozoite stages of wt and *Pbprkp*⁻ clones and analysed by western blotting. An antibody directed against a PbPKRP peptide from the predicted active site kinase domain detected a single, strong band in the wt parasites but not in the *Pbprkp*⁻ clones (Fig. 3B). The band corresponds to a protein of approximately 90 kDa, which is smaller than the protein predicted for PbPKRP (315.2 kDa). Gradient gels, specific for the detection of high molecular weight proteins, were also used but no additional bands were observed at any molecular weight (data not shown).

3.3. Reduced infection of mosquito midguts by *Pbprkp*⁻ parasites

We next characterized the role of PbPKRP in both the blood stage and mosquito stages of infection. Asexual growth in mice was unaffected in both *Pbprkp*⁻ clones and they gave rise to the same numbers of gametocytes as wt parasites (Supplementary Fig. S2A). Male gametes differentiated in vitro in both the wt and *Pbprkp*⁻ clones (Supplementary Fig. S2B). Mosquito infection, however, differed between the wt and *Pbprkp*⁻ parasites. We used a quantitative PCR assay to assess ookinete infection in the mosquito midgut. Twenty-four hours after feeding on mice infected with *Pbprkp*⁻ clones and wt parasites, the midguts of mosquitoes were assessed for parasite infection using specific primers for *Pb18S rRNA* and *Pbama-1*, together with primers to *Pbprkp* as a negative control. The amplification efficiency (Sikorsky et al., 2004; Yanow et al., 2006, 2008) of clones 1 and 2 was significantly reduced 64% and 71%, respectively, at the *Pb18S rRNA* locus and 58% and 65%, respectively, at the *ama-1* locus. There was no amplification of *prkp*, as expected (Fig. 4A). This suggests that fewer *Pbprkp*⁻ ookinetes were present in mosquito midguts 24 h after infection. However, since asexual parasites in the blood meal exceed the numbers of ookinetes that associate with the midgut, it is possible that even after repeated washing, a carry-over from lysed parasites could distort the result of the PCR analysis. To confirm the reduction of midgut infection, we also analyzed oocyst formation in the midgut at different times after feeding. We found a significant decrease in oocyst numbers in mosquitoes infected with *Pbprkp*⁻ clones compared with the wt (Fig. 4B).

A quantitative PCR assay was also used to determine the oocyst production in the mosquito midguts. Similar to the results at the ookinete stage, the amplification efficiency of DNA extracted from mosquito midguts on day 18 p.f. showed approximately an 80-90% reduction in parasite gDNA compared with wt parasites, suggesting fewer oocysts were established (Fig. 5A). Midgut infections were also assessed by sporozoite counts every second day from days 10 to 24 p.f.. Overall, the number of sporozoites in the midguts of the *Pbprkp*⁻ clone 1 and clone 2 was reduced 44% and 51%, respectively, throughout the course of infection (Fig. 5B), although this decrease was not significant due to the inherent variation in infection levels between experiments. Transmission electron microscopy images of wt and mutant oocysts within infected midguts did not reveal any morphological differences in the parasites (Fig. 5C).

These results suggest that the reduction in midgut sporozoites may arise from a defect in ookinete and oocyst production and indicates a role for PbPKRP prior to sporoblast formation in the endothelium of the mosquito.

3.4. *Pbpkp*⁻ sporozoites show a reduced capacity to invade mosquito salivary glands

The number of salivary gland sporozoites in wt and *Pbpkp*⁻ clones was assessed at the same time as midgut infections. A significant reduction in the number of salivary gland sporozoites was observed for both clones (75.7% for clone 1 and 76.2% for clone 2; Fig. 6A). This was further confirmed by quantitative PCR on DNA from salivary glands collected at day 18 p.f. As observed by sporozoite counts, there was a 75% reduction in amplification efficiency for both the *Pb18S rRNA* locus and the *ama-1* locus using salivary gland DNA from *Pbpkp*⁻ parasites. (Fig. 6B). Together, these results suggest that PbPKRP may be involved in multiple stages of parasite development in the mosquito. Interestingly, although there were fewer *Pbpkp*⁻ sporozoites inside the mosquito salivary glands, these sporozoites were not deficient in their subsequent infectivity. When equal numbers of *Pbpkp*⁻ salivary gland sporozoites were assessed for their gliding motility (see Supplementary Fig. S3A), hepatocyte invasion (see Supplementary Fig. S3B) and formation of exoerythrocytic forms in vitro (see Supplementary Fig. S3C), there was no difference compared with wt parasites. Finally, when equal numbers of salivary gland sporozoites were injected into mice i.v., the kinetics of asexual growth was similar to wt kinetics (data not shown).

Given the greater reduction of sporozoite numbers associated with the salivary glands compared with the midgut, it is possible that *Pbpkp*⁻ sporozoites have a reduced capacity to invade the salivary gland. The experiments shown in Fig. 6 detect both sporozoites attached to the outside of the gland as well as sporozoites within the gland. To test for the capacity of sporozoites to invade the salivary gland, whole glands were treated with trypsin to release sporozoites that had adhered to the outside of the glands. Sporozoites that were attached were located in the supernatant of treated, whole salivary glands, and the numbers were quantified. The number of sporozoites attached to the salivary glands in *Pbpkp*⁻ sporozoites was 57.0% and 86.6% reduced for clones 1 and 2, respectively, over the infection cycle compared with wt parasites (Fig. 7A). This reduction in attachment to the salivary gland does not account for the low numbers of sporozoites in salivary glands found in mosquitoes infected with *Pbpkp*⁻ sporozoites because it represents only about 3-8% of the total wt sporozoite numbers (Fig. 6A); however, it might indicate that the *Pbpkp*⁻ sporozoites have a reduced capacity to recognize the salivary gland for attachment.

An alternative hypothesis is that the *Pbpkp*⁻ sporozoites fail to migrate from the midgut epithelium to the salivary gland. Sporozoites emerging from the oocyst would be released into the circulation in the mosquito hemocoel, which sporozoites use to transit to the salivary glands. The number of *Pbpkp*⁻ sporozoites circulating in mosquito hemocoel was not significantly different from wt parasites (Fig. 7B), indicating that sporozoites did migrate out of the midgut into the hemocoel. Given the fact that the number of midgut sporozoites is reduced, one would also expect that the number of hemocoel sporozoites would be similarly reduced, but the numbers are very similar, indicating that many *Pbpkp*⁻ sporozoites are trapped and accumulate over time within the hemocoel. We propose that PbPKRP affects the numbers of ookinetes and oocysts, which in turn affects the number of sporozoites in the mosquito midgut. Those sporozoites that do successfully develop are able to migrate to the mosquito hemocoel but are less able to infect mosquito salivary glands than wt parasites, resulting in fewer salivary gland sporozoites.

4. Discussion

CaM is a ubiquitous, calcium-binding protein that can bind and regulate a multitude of different protein targets, thereby affecting many different cellular functions (Silva-Neto et al., 2002). CaMKs are primarily regulated by a change in Ca^{2+} concentrations. In this study, we show that a CaMK-related protein (PbPKRP) has an important function in mediating the level of *P. berghei* infection in *A. stephensi* mosquitoes. The analysis of the *pkrp*⁻ deletion mutants shows that this protein is not essential for asexual and sexual intraerythrocytic development. However, PbPKRP seems to be involved in mediating the infection of ookinetes in the midgut epithelium of the mosquito, thereby affecting the number of oocysts formed and resulting in decreased sporozoite production. In addition, sporozoites that are released into the mosquito hemocoel have a reduced ability to invade the salivary glands. The results suggest that PbPKRP influences the potential transmissibility of malaria by each mosquito to another mammalian host.

Assigning clear orthology of PbPKRP to a human protein kinase is difficult, given the phylogenetic distances between the species. Based on bioinformatic analysis, PbPKRP is most closely related to the human enzyme TSSK1. Human TSSK1 is almost exclusively expressed in the testes, and a role for this enzyme in the fusion of the sperm and oocyte, together with the breakdown of the nuclear envelope, has been proposed (Hao et al., 2004). Interestingly, this is consistent with our data suggesting that PbPKRP plays a role in cell invasion. As is true with TSSK1, PbPKRP shows sequence similarity with the CaMK family, although it contains no EF-hand motifs, and has a conserved catalytic aspartate. The protein is predicted to be an atypical kinase with a single glycine in the glycine loop motif. Although numerous attempts were made to clone and express an active form of PbPKRP in *Escherichia coli*, none were successful (data not shown). Expression of *Plasmodium* proteins has proven to be challenging, with estimates that only 6.3% of attempts at heterologous expression are successful (Mehlin et al., 2006).

The native PbPKRP was identified as a protein of about 90 kDa. It is likely that PbPKRP is processed at the protein level. Post-translational and/or post-transcriptional processing is well-characterized in protein kinases, sometimes occurring in a tissue-specific manner (Kosaka et al., 1988; Bruggemann et al., 2000). This processing could play a role in suppressing kinase activity until the ookinete is formed and is ready to invade the mosquito endothelium. Alternatively, the gene prediction may not be accurate and the PbPKRP transcript which gets translated may be smaller than predicted. Further work is needed to resolve this question, such as generating antibodies to the predicted C terminal region of PbPKRP to determine whether a larger precursor form of PbPKRP is expressed.

The mechanisms underlying the invasion of the ookinete into the mosquito midgut, exit of the sporozoites from the midgut and sporozoite entry into the salivary glands are not well characterized. Given the quantitative reduction in *Pbpkrp*⁻ parasites at the salivary gland stages, PbPKRP appears to be involved in the invasion of this tissue. There is also a clear reduction in midgut stages, however we have not determined whether PKRP is necessary for midgut invasion or early oocyst development. Some evidence indicates that parasite interactions with salivary glands and with midgut are specific (Ghosh et al., 2001; Han and Barillas-Mury, 2002), but little is known about the parasite molecules that interact with these tissues. A small peptide, called SM1, has been shown to bind to the luminal side of the midgut epithelium and the distal lobes of the salivary glands and interfere with parasite infection in mosquitoes. SM1 presumably blocks a specific interaction between a receptor on the parasite and a binding site on the midgut epithelium and the distal lobes of the salivary glands. Interestingly, this peptide does not bind to the midgut surface in the hemocoel (Ghosh et al., 2001; Ito et al., 2002). Development of transgenic mosquitoes that express inhibitory molecules or with disrupted ligands has been proposed as a method to control malaria transmission by

replacing wt mosquitoes with transgenic mosquitoes that are refractory to infection (James, 2003). The identification of PKRP as a regulator of parasite development in the mosquito is a further step toward characterizing protein interactions that could lead to the development of compounds with transmission-blocking potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Alignments of calmodulin-dependent protein kinase (CaMK) and putative kinase related protein (PKRP) sequences. (A) The first 500 amino acids of the CaMK-related proteins found in four *Plasmodium* spp. are aligned. Amino acids found in two of the four aligned sequences are shaded to show identity. The vertical bars at residues 161 and 441 indicate the N- and C-terminal boundaries of the catalytic domain, respectively. The sequence of the peptide used for immunization is indicated by the horizontal bar. Residues that are largely conserved in Ser/Thr protein kinases are indicated above the *Plasmodium falciparum* (Pf)PKRP sequence. The residues indicated at the top are: a glycine (G), which constitutes part of the glycine triad, the lysine (K), which anchors and orients ATP, a glutamate (E) that forms a salt bridge, the eukaryotic protein kinase signature motif (RDxxxxN), the Mg²⁺-binding motif (DxG), the glutamate (E) and aspartate that provide structural stability, and a conserved arginine (R). (B) The catalytic domains of PfPKRP and *Plasmodium berghei* (Pb)PKRP were aligned with

human TSSK1 as an example of a related CaMK protein kinase. Pc, *Plasmodium chabaudi* ; Py, *Plasmodium yoelii* ; h, human.

Fig. 2. Targeted disruption of the putative kinase related protein gene in *Plasmodium berghei* (*Pbprp*). (A) Schematic representation of the *Pbprp* locus and the gene-targeting construct used for gene replacement by double homologous recombination, together with the resulting disrupted locus. (B) Diagnostic PCR verifying the disruption of the *Pbprp* locus. Representative PCR is shown using primers to the wild-type (wt; clone C₁) open reading frame (labeled “wt” in A), a PCR of the mixed wt and transfected parasites which contain genomic DNA (gDNA) that show products from the wt and 5’ and 3’ integrated primers (labeled “5’int” and “3’int” in A). Finally, gDNA from a representative disrupted clone (clone 1) shows only the 5 and 3’ integrated products. A negative control (-ctrl; gDNA alone) was also included. (C) Southern blot analysis of *BglII*-digested DNA, showing the intact *pkrp* locus in the wt

population and a shifted, smaller band associated with the disrupted loci of both clone 1 and 2.

Fig. 3.

Northern and western blot analyses of putative kinase related protein (PKRP) expression in wild-type (wt) and *pkrp*⁻ sporozoites. (A) Total RNA was extracted from wt and *pkrp*⁻ sporozoites and 5 µg of RNA from each clone were used for northern blot analyses: the blots were probed with a labeled product of the primer set ORF F/ORF R labeled in Fig. 2A. (B) Ethidium bromide-stained agarose gel showing equal loading of total RNA. (C) Western blot analyses were completed on protein extracts from 7.5×10^4 wt and *pkrp*⁻ sporozoites. The blots were probed with the anti-peptide rabbit polyclonal serum raised against a conserved kinase domain of PKRP (see Fig. 1A).

Fig. 4.

Evaluation of midgut invasion and oocyst formation by ookinetes lacking the gene for putative kinase related protein (*pkrp*⁻). *Anopheles stephensi* mosquitoes were fed directly on groups of five (A) or three (B) mice infected with wild-type (wt) or *pkrp*⁻ (both clones 1 and 2) parasites with matched parasitemias and gametocytemias on day 0. (A) At 24 h after feeding, midguts were dissected from 10 mosquitoes from each group. The blood meal was removed and the midgut was washed extensively. Genomic DNA was extracted from the pooled midguts from each group. Malaria infection was determined using quantitative PCR and primers directed towards *Plasmodium berghei* (*pb*) *pb18s rRNA* (white bars), *pbama-1* (grey bars) and *Pbpbkrp* (black bars). The experiment was performed twice using independent parasite cycles and each sample was run in triplicate for each quantitative PCR (qPCR) cycle. The average of the results of two qPCRs with the standard error (SE) is shown. * The amplification efficiency was significantly reduced ($P < 0.0001$, ANOVA, $n = 10$) in both clones of *pkrp*⁻ ookinetes compared with wt ookinetes. (B) At different days after feeding, the total number of oocysts

in each midgut was counted in 20 mosquitoes from each group. The number of oocysts was significantly decreased ($P < 0.0001$, two-way ANOVA) in midguts fed on clone 1 and clone 2 compared with wt.

Fig. 5.

Evaluation of sporozoite production and morphology in midguts of mosquitoes infected with clones lacking the gene for putative kinase related protein (*pkrp*⁻). (A) *Anopheles stephensi* mosquitoes were fed directly on mice infected with wild-type (wt) or *pkrp*⁻ (both clones 1 and 2) parasites on day 0. On day 10 post-feeding (p.f.), mosquito midguts were dissected from 10 mosquitoes from each group and assayed using the quantitative PCR technique as shown in Fig. 4. There was a significant reduction (*) in amplification efficiency in *pkrp*⁻ DNA for the *pb18s rRNA* (white bars) and *pbama-1* (grey bars) genes in mosquito midguts ($P < 0.0001$, ANOVA, $n = 10$). There was no amplification of the *Pbpkrp* gene (black bars). The infection was repeated twice using independent parasite cycles and each sample was run in triplicate for each qPCR cycle. The average of the results of two qPCRs from each independent cycle with the standard error (SE) is shown. (B) Every second day from day 10 to day 24 p.f., midguts were dissected from 10 mosquitoes from each group. The number of sporozoites per mosquito was determined and the total number of sporozoites (expressed as area under the curve, AUC)

calculated for the duration of the infection. A reduction in the number of sporozoites in the midguts of mosquitoes fed on *pkrp*⁻ parasites persisted throughout the mosquito infection. Shown is the mean AUC with the SE for the number of sporozoites per mosquito in four independent experiments using individual batches of mosquitoes, with 80 mosquitoes dissected in each of the independent experiments. (C) Representative transmission electron micrograph of oocyst sporozoites of a mosquito fed on mice infected with wt parasites. Electron micrographs of representative oocyst sporozoites of mosquitoes fed on mice infected with clones 1 and 2 of *Pbpkp*⁻ parasites. N = nucleus; M = microneme; R = rhoptry. Scale bar represents 2 μ m.

Fig. 6.

Evaluation of sporozoite numbers in the salivary glands of mosquitoes infected with clones without the gene for putative kinase related protein (*pkrp*⁻). (A) *Anopheles stephensi* mosquitoes were fed directly on mice infected with wild-type (wt) or *pkrp*⁻ (both clones 1 and 2) parasites on day 0. Every second day from day 10 to day 24 post-feeding (p.f.), salivary glands were dissected from 10 mosquitoes from each group. The number of sporozoites per mosquito was determined and the total number of sporozoites (expressed as area under the curve, AUC) calculated for the duration of the infection. A significant reduction (*) of *pkrp*⁻ salivary gland sporozoites was observed throughout the infection ($P < 0.0001$, ANOVA, $n = 400$). Shown is the mean AUC with the standard error (SE) for the number of sporozoites per mosquito in four independent experiments using individual batches of mosquitoes, with 80 mosquitoes dissected in each of the independent experiments. (B) On day 18 p.f., mosquito salivary glands were dissected from 10 mosquitoes from each group. Genomic DNA was extracted from the pooled midguts from each group. Malaria infection was determined using quantitative PCR and primers directed towards *pb18s rRNA* (white bars), *pbama-1* (grey bars) and *Pbpbkrp* (black bars). There was a significant reduction (*) in amplification efficiency in salivary glands from mosquitoes infected with *pkrp*⁻ parasites ($P < 0.0001$, ANOVA, $n = 10$). Results are the average with the SE of two independent experiments with 10 mosquitoes per group per clone.

Fig. 7.

Evaluation of sporozoite numbers attached to the salivary glands and circulating in hemocoel of mosquitoes infected with putative kinase related protein (*pkrp*⁻) deficient parasites. *Anopheles stephensi* mosquitoes were fed directly on mice infected with wild-type (wt) or *pkrp*⁻ (both clones 1 and 2) parasites on day 0. On day 10 to day 24 post-feeding (p.f.), hemocoel was collected from 10 mosquitoes from each group. On the same days, whole salivary glands were dissected from the same number of mosquitoes and treated with trypsin to detach sporozoites on the outside of the glands. (A) The number of sporozoites attached to salivary glands per mosquito was determined and the area under the curve (AUC) calculated for the duration of the infection. Fewer sporozoites were attached to mosquito salivary glands in parasites lacking the *pkrp*⁻ locus. A significant reduction (*) of *pkrp*⁻ sporozoites attached to salivary glands was observed throughout the course of infection ($P = 0.0081$, ANOVA, $n = 400$). The error bar corresponds to the standard error (SE) of three independent experiments. (B) The number of hemocoel sporozoites per mosquito was equivalent in both the wild-type and *pkrp*⁻ parasites. Shown is the average AUC and the SE of the sporozoite numbers calculated for the duration of the experiment from three independent experiments.