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Plasmodium P47: a key gene for malaria transmission by mosquito vectors

Alvaro Molina-Cruz[1,*], **Gaspar E. Canepa**[1], and **Carolina Barillas-Mury**[1,*]

[1]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852

Abstract

Malaria is caused by infection with *Plasmodium* parasites that have a complex life cycle. The parasite protein P47 is critical for disease transmission. P47 mediates mosquito immune evasion in both *Plasmodium berghei* (Pbs47) and *Plasmodium falciparum* (Pfs47), and has been shown to be important for optimal female gamete fertility in *P. berghei*. *Pfs47* presents strong geographic structure in natural *P. falciparum* populations, consistent with natural selection of *Pfs47* haplotypes by the mosquito immune system as the parasite adapted to new vector species worldwide. These key functions make *Plasmodium P47* an attractive target to disrupt malaria transmission.

Keywords

malaria transmission; *Plasmodium* fertilization; *Plasmodium falciparum*; *Plasmodium berghei*; immune evasion; P47; Pfs47; mosquito immunity; complement-like system; female gamete

Introduction

Malaria is the most important human parasitic disease, with 212 million cases and 429,000 deaths in 2015 [1]. It is caused by *Plasmodium* parasites with a complex life cycle that alternates between a vertebrate host and a mosquito vector. *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent agents of human malaria and are transmitted by anopheline mosquitoes. While *Plasmodium* parasites have a rather restricted vertebrate host range, they have adapted to at least 70 different mosquito species [2], many of them evolutionarily distant from vectors in Africa, where human malarias originated [3,4].

Plasmodium undergoes obligatory sexual reproduction and multiple developmental stages in the mosquito [5–7]. Mosquitoes become infected when a female ingests a blood meal containing *Plasmodium* gametocytes (Fig 1). These develop into gametes in the mosquito midgut lumen and fuse to form a zygote, which subsequently matures into a motile ookinete and invades the mosquito midgut epithelium. If the ookinete succeeds in traversing the

*Correspondence should be addressed to: cbarillas@niaid.nih.gov (CB-M) and amolina-cruz@niaid.nih.gov (AM-C).

midgut epithelial cell, it transforms into an oocyst and replicates, generating thousands of sporozoites that are released into the mosquito hemolymph. Some sporozoites are able to invade the salivary gland and are transmitted to another person when the mosquito acquires a subsequent blood meal. To be transmitted, *Plasmodium* parasites must overcome many obstacles [8], such as physical barriers and antiplasmodial responses that target ookinetes [9] or oocysts [10,11]. In some incompatible parasite-vector combinations, the mosquito complement-like immune response eliminates most ookinetes [9,12,13].

Ookinete midgut invasion activates a strong epithelial nitration response [14,15] that triggers local release of hemocyte-derived microvesicles [16] that, in turn, promote mosquito complement-like activation. The *Anopheles gambiae* thioester containing protein (TEP1), a homolog of complement factor C3 in vertebrates [17], is stabilized in the hemolymph by interacting with the leucine-rich repeat proteins LRIM1 and APL1C [18,19] (Fig. 1). When activated, TEP1 binds to the ookinete surface and triggers the formation of a complex that kills the parasites. Here we review the known biological functions of *P47* critical for malaria transmission: *Plasmodium* fertilization and parasite evasion of the mosquito immune system. The importance of immune evasion for the adaptation of *Plasmodium falciparum* to different mosquito species during the globalization of malaria will be discussed.

P47 organization

P47 is one of 14 members of the six-cysteine (6-Cys) protein family [20]. The characteristic 6-Cys domain (also called s48/45 domain) was initially identified in Pfs230 [21]. Although the protein sequence homology is low (14–36% amino acid identity), this family is characterized by containing anywhere from 1 to 14 copies of the 6-Cys motif domain, and have clear orthologs in all *Plasmodium* species that have been analyzed. Members of the 6-Cys family are secreted, or membrane-anchored proteins, and are expressed at different stages of the parasite's life cycle. Some of them are important for sporozoite liver invasion, while others are involved in fertilization [20] or mosquito immune evasion [22].

The *P47* gene was initially identified in *P. falciparum* (*Pfs47*) based on sequence homology to other 6-Cys members, such as Pfs230 and Pfs48/45, two of the leading transmission-blocking vaccine targets [23–25]. The *Pfs47* (PF3D7_1346800) gene is localized in chromosome 13, adjacent to Pfs48/45 (PF3D7_1346700). Both genes lack introns and have a similar domain organization, consisting of three 6-Cys domains, yet share low sequence homology (26% amino acid identity). Pfs48/45 is expressed on the surface of both male and female gametocytes and gametes, and is required for male fertility [26].

Pfs47 has a signal peptide and a putative GPI anchor sequence (Fig. 2), domains 1 and 3 are characteristic 6-Cys domains, while domain 2 is a degenerate s48/45 domain with only two cysteines (Fig 2) [27]. Although there are clear Pfs47 orthologs in other *Plasmodium* species, the sequence homology is low (Fig. S1). For example, *P. falciparum* and *P. vivax* have 42% amino acid identity. Analysis of *P47* sequences from several rodent malaria parasites indicates that *P47* is evolving under positive selection [28,29] with exceptionally high ratio of nonsynonymous to synonymous substitutions (dN/dS) in the second domain [28].

Biological function of P47

Fertilization

P47 is localized on the surface of female gametocytes (Fig. 1) and gametes, as well as of zygotes and ookinetes [22,30,31]. It does not seem to be essential for fertilization in *P. falciparum* because gametocyte cultures in which the *Pfs47* gene was disrupted (Pfs47-KO) efficiently infected *A. stephensi* Nijmegen mosquitoes [31]. Furthermore, three anti-Pfs47 monoclonal antibodies did not inhibit *A. stephensi* infection with wild-type *P. falciparum* parasites [31]. Later studies in *P. berghei* found that *P47* was required for female fertility under *in vitro* culture conditions, and disruption of the gene also significantly impaired fertilization *in vivo* [28]. Although *Pfs47* is not essential for fertilization when mosquitoes are fed large number of cultured *P. falciparum* gametocytes, it may play an important role in optimizing fertilization under *in vivo* conditions, as observed in the *P. berghei* system, in which much smaller numbers of gametocytes are ingested by female mosquitoes.

Immune evasion

An *A. gambiae* strain (L3–5), genetically selected to be highly refractory to *P. cynomolgi* infection, also eliminated most *P. falciparum* strains from Asia or the Americas but, interestingly, some parasite strains from West Africa survived infection of the mosquito [32]. Later studies showed that the African strains that survive, evade the mosquito complement-like system [13]. A genetic cross between the Brazilian 7G8 *P. falciparum* strain that is eliminated and the African GB4 strains that survives in *A. gambiae* L3–5, was used to identify the gene that made the African parasites “invisible” to the mosquito immune system. A combination of genetic mapping, linkage group selection and functional genetics identified *Pfs47* as a gene required for *P. falciparum* to evade immune detection [22]. There are only four amino acid differences between the Pfs47 proteins in the 7G8 and GB4 strains (Fig. 2), all present between the two cysteines in domain 2, that are key determinants of parasite survival in *A. gambiae* L3–5 [22,33]. In contrast, both parasite lines (GB4 and 7G8) readily infect the *A. gambiae* G3 strain, indicating that mosquito genetic factors also determine how effective different Pfs47 haplotypes are in promoting evasion of mosquito immunity [22]. It is clear, however, that *Pfs47* greatly enhances *P. falciparum* parasite survival in both *A. gambiae* G3 and L3–5 strains, because the great majority of *P. falciparum* (NF54) parasites in which the *Pfs47* gene has been disrupted are readily eliminated in both mosquito strains [22]. *Pfs47* appears to prevent elimination of the parasite by disrupting c-Jun N-terminal kinase (JNK) signaling [34], a pathway that is essential to trigger epithelial nitration [34,35]. Lack of nitration precludes the release of hemocyte-derived microvesicles [16] and prevents local TEP1 activation and binding on the ookinete surface [22,34]. Interestingly, a Pfs47-KO line readily infects the *A. stephensi* (Nijmegen strain) mosquitoes [31] [22]. It is possible that because the *A. stephensi* Nijmegen strain was genetically selected for high infectivity with *P. falciparum* [36], this colony was fixed for some polymorphism that disrupts antiplasmodial immunity. Alternatively, some vector species may be naturally very permissive to many different P47 haplotypes.

Similar to Pfs47, recent studies show that *Pbs47* is also required for *P. berghei* to evade the complement-like system in *A. gambiae* [37]. Interestingly, *P. berghei* is particularly

susceptible to melanization by *A. gambiae* mosquitoes carrying the TEP1-R1 allele [9], but this is not the case for *P. falciparum*. For example, when the G3 (TEP1 S3/S3) and L3–5 (TEP1 R1/R1) mosquitoes were mixed in a hybrid colony and allowed to mate for many generations, there was a strong association between *P. berghei* elimination and the TEP R1 allele, with melanization frequencies of 98% in TEP1 R1/R1 homozygous, 73% in R1/S3 hybrids and 10% in S3/S3 females. In contrast, *P. falciparum* 7G8 parasites, that are very effectively melanized by the L3–5 strain, were not melanized at all by mosquitoes of this hybrid colony [13]; indicating that, besides TEP1 R1, there are other gene(s) in L3–5 mosquitoes that are required to trigger *P. falciparum* melanization. However, it is possible that TEP1 R1 could enhance lysis of *P. falciparum* 7G8 parasites. For example, a genetic cross between a *A. gambiae* M colony fixed for TEP1r^B (Mali-NIH) and one for fixed for TEP1s (Yaoundé) showed a similar dominant effect of TEP1 r^B on *P. berghei* melanization and lack of melanization of the ND37 clone obtained from *P. falciparum* NF54. The number of ND37 oocysts was significantly lower (a reduction of about 40% in the mean number of oocysts) in TEP1s/r^B compared to TEP1 s/s females, suggesting that TEP1 r^B, or some gene in close proximity, promotes *P. falciparum* lysis. The effect of TEP1 r^B in *P. falciparum* is less dramatic than when the same mosquitoes are infected with *P. berghei* [38].

P47 population structure and selection by mosquito vectors

Population structure studies of *Pfs47*, based on a limited number of laboratory and field isolates, revealed a strong geographic structure [39], similar to what had previously been described for Pfs48/45 [40]. Genotyping of 35 *P. falciparum* oocysts from field-infected *A. gambiae* from Tanzania showed high inbreeding coefficients for *Pfs47* and *Pfs48/45* suggestive of assortative mating; while *Pfs47* single nucleotide polymorphism (SNP) analysis revealed a modest, but significant, difference in the *Pfs47* haplotypes present in field-infected *A. gambiae* vs. *A. funestus* mosquitoes. This suggests that *Pfs47* is under natural selection by these two vectors [39]. Furthermore, *Pfs47* is one of the *P. falciparum* genes with the highest SNP differentiation between Africa, Asia and Oceania (based on whole genome population genetic analysis of 227 isolates) [41]. *P. vivax* P47 (*Pvs47*) is also polymorphic [42], and is one of the genes with the highest population differentiation between continents (based on whole genome sequences of 195 isolates) [43]. Analysis of 516 Cambodian isolates detected 22 different Pfs47 protein haplotypes closely-related to all other Asian isolates [44], in agreement with clustering of certain haplotypes in different continents.

Consistent with these previous reports, analysis of 364 *Pfs47* sequences from *P. falciparum* isolates collected around the world identified 47 DNA haplotypes that exhibit a high dN/dS, suggestive of natural selection [45]. The 42 Pfs47 protein sequence haplotypes identified, share 97.7–99.8% amino acid identity, with Domain 2 being the most polymorphic region of the protein. Phylogenetic analysis showed that the haplotypes cluster into two main clades. The largest clade includes 32 haplotypes that are more frequent in Africa, one exclusive to Papua New Guinea and three that are the only ones detected in the Americas, consistent with the African origin of *P. falciparum* [46]. The smaller clade, includes six haplotypes that are frequent in Asia but were not detected in the Americas. In summary, both *Pfs47* and *Pvs47* haplotypes present a marked geographical population structure at a continental level not

observed in most other genes, suggesting that this population structure is the product of a natural selection process that favors certain *P47* haplotypes in a given continent [45].

Immune evasion and globalization of *P. falciparum* malaria

Direct comparison of the compatibility between three major malaria vectors from Africa (*A. gambiae*), Southeast Asia (*A. dirus*) and the Americas (*A. albimanus*), with *P. falciparum* isolates collected from these continents, supports the hypothesis that *Pfs47* has been important for the adaptation of *P. falciparum* to evolutionarily distant Anopheline vectors. Anopheline mosquitoes had higher compatibility (i.e. higher infection intensity and prevalence) when infected with *P. falciparum* from the same geographic region, suggesting that *P. falciparum* underwent natural selection while adapting to different vectors [45]. The mosquito immune system was shown to be a major determinant of parasite-vector compatibility, because disruption of the mosquito complement system greatly enhanced infection in combinations with low compatibility. Furthermore, genetic replacement of the *Pfs47* haplotype in an African *P. falciparum* line with *Pfs47* haplotypes from other geographic regions was sufficient to change the compatibility with these anopheline vectors by allowing the parasite to evade the mosquito complement-like system [45]. Taken together, these studies indicate that the mosquito immune system has been an important barrier for adaptation of *P. falciparum* to distant anopheline species through selection of *Pfs47*, which may have influenced the parasite's population structure and the epidemiology of malaria. Based on these findings, the "lock-and-key theory" of *P. falciparum* globalization was proposed, in which Pfs47 is the "key" that interacts with a mosquito receptor ("lock"), disrupting the antiplasmodial response [45]. The receptors are predicted to be different in evolutionary distant vectors and to select parasites that carry a compatible Pfs47 haplotype (the correct key), by allowing them to evade immune detection.

This model has important implications for the spread of the kelch propeller domain protein (K13) mutations that mediate delayed parasite clearance in response to artemisinin, because the *K13* and *Pfs47* genes are likely to be genetically linked, due to their close proximity (151 kb) [47]. *P. falciparum* lines with these K13 mutations are associated with the two most frequent *Pfs47* haplotypes in Asia [44]. African mosquito vectors can be infected with these lines [44,45], but the level of infection in *A. gambiae* is significantly lower than in *A. dirus* (Asian) mosquitoes [45]. This would suggest that the K13 mutations could readily spread from Asia to Africa if the parasite is under drug pressure, which was the case for the spread of chloroquine resistance from Asia to Africa [48]. These Asian lines have very low compatibility with *A. albimanus* (New World vector), suggesting that the K13 mutations are less likely to spread from Asia to the New World [45].

Infections of *A. gambiae* (Ngousso and L3–5 strains) with three different African *P. falciparum* isolates, showed that most parasites from two of the isolates survived in both mosquito strains. However, most parasites from the third isolate (NF165) were eliminated by the mosquito complement-like system in both the Ngousso and L3–5 strains [49]. This was unexpected, because the predicted protein sequence of Pfs47 Domain 2 from NF165 is identical to that of Pfs47 from GB4, a strain that evades mosquito immunity. Two potential explanations for this discrepancy could be that NF165 may have other amino acid

differences outside Domain 2 (only 59% of the *Pfs47* gene was sequenced) that may be major determinants of compatibility in some parasite strains, or that, besides *Pfs47*, other genes may be also be required for effective immune evasion. Although the great majority of Pfs47-KO parasites are eliminated by the mosquito complement-like system in both G3 and L3–5 mosquitoes, the fact that some Pfs47-KO parasites can infect *A. gambiae* mosquitoes indicates that *Pfs47* is not absolutely essential for parasite survival [22, 45].

Although it is apparent that the immune system of some anophelines selects certain Pfs47 haplotypes, there is no evidence of fertilization incompatibility between *P. falciparum* strains from different continents, as genetic crosses readily produce hybrid progeny [50,51]. The human immune system is another possible selective force on *Pfs47*, as gametocytes not ingested by mosquitoes can elicit immunity [52,53], and antibodies following immunization with recombinant *P. vivax* Pvs47 have transmission-blocking activity [54]. This has led us to re-evaluate *Pfs47* as a potential transmission-blocking vaccine target.

Conclusions

Plasmodium P47 is critical for successful malaria transmission. In *P. berghei*, *P47* is required for optimal fertilization, but in *P. falciparum* this requirement is not well established. In both *P. falciparum* and *P. berghei*, *P47* is also important for mosquito immune evasion. *P47* has one of the strongest signatures of natural selection and population structure in the *P. falciparum* and *P. vivax* genomes. The immune system of mosquitoes from different continents appears to be one of the forces that have selected *Pfs47* haplotypes as malaria became global. It is unclear whether all anopheline species exert selection on the parasite. The nature of the mosquito Pfs47 receptor, and the mechanism by which it disrupts JNK signaling also remain to be determined. The importance of *P47* for malaria transmission warrants further studies to assess its feasibility as a target to block malaria transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The *Plasmodium* P47 protein is critical for successful malaria transmission.
- In *P. berghei*, P47 is required for optimal fertilization.
- P47 is also important for mosquito immune evasion in *P. falciparum* and *P. berghei*.
- P47 has a strong geographic structure in *P. falciparum* and *P. vivax* populations.
- The mosquito immune system selects *Pfs47* haplotypes in different continents.

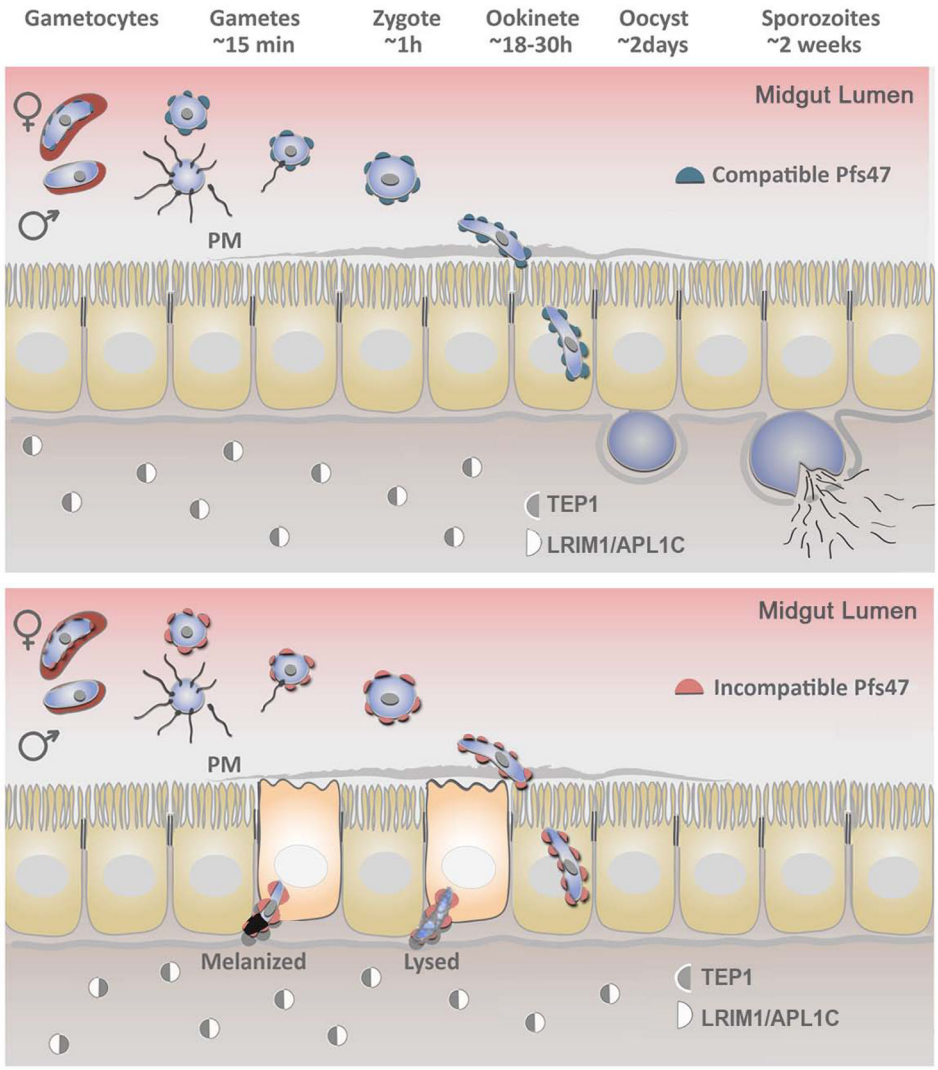

Figure 1. Role of *Plasmodium P47* in parasite development in the mosquito

Plasmodium infection of the mosquito is initiated when the mosquito takes a blood meal containing gametocytes. These gametocytes mature into gametes in the gut lumen and, within minutes, fuse to form a zygote. *P47* is expressed of the surface of female gametocytes and gametes, and in *P. berghei* it is required for optimal fertilization. The zygote develops into a motile ookinete that, one day after blood feeding, traverses mosquito midgut epithelial cells. *P47* is also expressed on the surface of ookinetes, and in *P. falciparum* and *P. berghei*, it allows parasite evasion of the mosquito immune system. Vector-compatible Pfs47 haplotypes (dark blue) inhibit JNK signaling and the induction of two key enzymes, NADPH-oxidase 5 (NOX5) and heme-peroxidase 2 (HPX2), that potentiate epithelial nitration in the invaded midgut cell. Successful ookinetes reach the basal membrane (BM) and form an oocyst. About two weeks later, oocysts release thousands of sporozoites into the hemolymph. Some sporozoites invade the salivary gland and are injected into a new vertebrate host when the mosquito takes another blood meal. Parasite lines that carry a vector-incompatible Pfs47 haplotype (red) trigger the midgut protein nitration response

leading to detection and binding of the thioester-containing protein TEP1 to the parasite surface. TEP1 forms a complex that eliminates the parasite through lysis or melanization. TEP1 is stabilized in the hemolymph by leucine-rich repeat proteins LRIM1 and APL1C. PM, Peritrophic matrix.

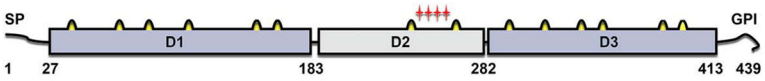

Figure 2.

Structural organization of *Plasmodium falciparum* Pfs47 protein (drawn to scale). Pfs47 has three domains (D1–D3). The D1 and D3 domains have 6 cysteines (in yellow), while the D2 domain only has 2 cysteines. Red stars denote the four amino acid differences between the West African *P. falciparum* line (GB4) that evades the immune system of *A. gambiae* L3–5 mosquitoes and the Brazilian strain (7G8) that is eliminated. SP, predicted signal peptide; GPI, glycosylphosphatidylinositol-anchoring signal; numbers indicate amino acid position.