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A Central Role for P48/45 in Malaria Parasite Male Gamete Fertility

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Summary

Fertilization and zygote development are obligate features of the malaria parasite life cycle and occur during parasite transmission to mosquitoes. The surface protein PFS48/45 is expressed by male and female gametes of *Plasmodium falciparum* and PFS48/45 antibodies prevent zygote development and transmission. Here, gene disruption was used to show that Pfs48/45 and the ortholog Pbs48/45 from a rodent malaria parasite *P. berghei* play a conserved and important role in fertilization. *p48/45*$^-$ parasites had a reduced capacity to produce oocysts in mosquitoes due to greatly reduced zygote formation. Unexpectedly, only male gamete fertility of *p48/45*$^-$ parasites was affected, failing to penetrate otherwise fertile female gametes. P48/45 is shown to be a surface protein of malaria parasites with a demonstrable role in fertilization.

Introduction

The malaria parasite (*Plasmodium* spp.) must infect its Anopheline mosquito vector to undergo transmission between vertebrate hosts. A developmental pathway is initiated when the mosquito ingests the specialized sexual precursor cells (gametocytes) of the parasite that circulate in the blood of an infected host. Within the mosquito, these gametocytes rapidly differentiate to produce male and female gametes that fertilize and form zygotes. The zygote develops within the blood meal into a motile form, the ookinete, which traverses the midgut wall forming an oocyst on the hemolymph side of the midgut to establish the parasitic infection of the vector. Sex is, therefore, an obligatory part of the life cycle of the malaria parasite.

The central role of zygote formation in the life cycle and transmission of the parasite makes gametes and zygotes attractive targets for interruption strategies to prevent transmission of the disease. An actively pursued approach to block transmission of malaria parasites is immunization of the human host with parasite proteins that will generate a transmission-blocking immune response principally through the action of antibodies. Various surface proteins of the gametes and zygotes have been proposed as candidate antigens for the development of transmission-blocking components of a vaccine against *P. falciparum*, for example, Pfs48/45, Pfs230, and Pfs25/28 (Kaslow et al., 1988; Kocken et al.,1993; Williamson et al., 1993; Duffy and Kaslow, 1997). Immune responses elicited by vaccination against these proteins have successfully targeted events during sexual development of the parasite, resulting in reduction of transmission capacity (Kaslow, 1996; Carter et al., 2000).

Antibodies against Pfs48/45 cause a significant reduction of transmission due to the inhibition of zygote development and mosquito infection (Vermeulen et al., 1985; Carter et al., 1990; Targett et al., 1990; Roeffen et al., 1996). Pfs48/45 is specifically expressed in gametocytes and gametes and is thought to be anchored at the parasite surface by a glycosylphosphatidylinositol linkage (Vermeulen et al., 1986; Kocken et al., 1993). It contains two copies of a six-cysteine (6-cys) structural domain, unique to *Plasmodium*, and is a member of a gene superfamily encoding proteins that share an overall conserved structure based upon these 6-cys domains (Carter et al., 1995; Templeton and Kaslow, 1999). Blast analysis of sequences present in the *P. falciparum* genome sequencing project has identified nine proteins in this family to date, including Pf12, Pf47, and Pfs230 and its paralog (J. T., unpublished data; Elliott et al., 1990; Templeton and Kaslow,1999). Pfs230, like Pfs48/45, is present in gametocytes and gametes and appears to physically interact with Pfs48/45 (Kumar, 1987). The expression pattern and precise function of other members of the P48/45 superfamily is currently unknown. Determination of the essential nature, function, and interaction of Pfs48/45 and other proteins involved in the sexual cycle is crucial for a rational approach to improve and develop effective transmission-blocking components for a malaria vaccine (Kaslow, 1996).

Genetic modification of malaria parasites is now an established approach to examine the function of proteins and has particular application in *P. berghei* to study genes expressed in the mosquito stages of parasite development (Dessens et al., 1999; Wengelnik et al., 1999; Ménard, 2000). In this study, we disrupted the *pfs48/45* of *P. falciparum* and isolated and disrupted the orthologous gene (Pbs48/45) of *P. berghei.* Comparison of the phenotype of knockout (ko) parasites of both species demonstrated a central and conserved role for P48/45 in male gamete fertility.

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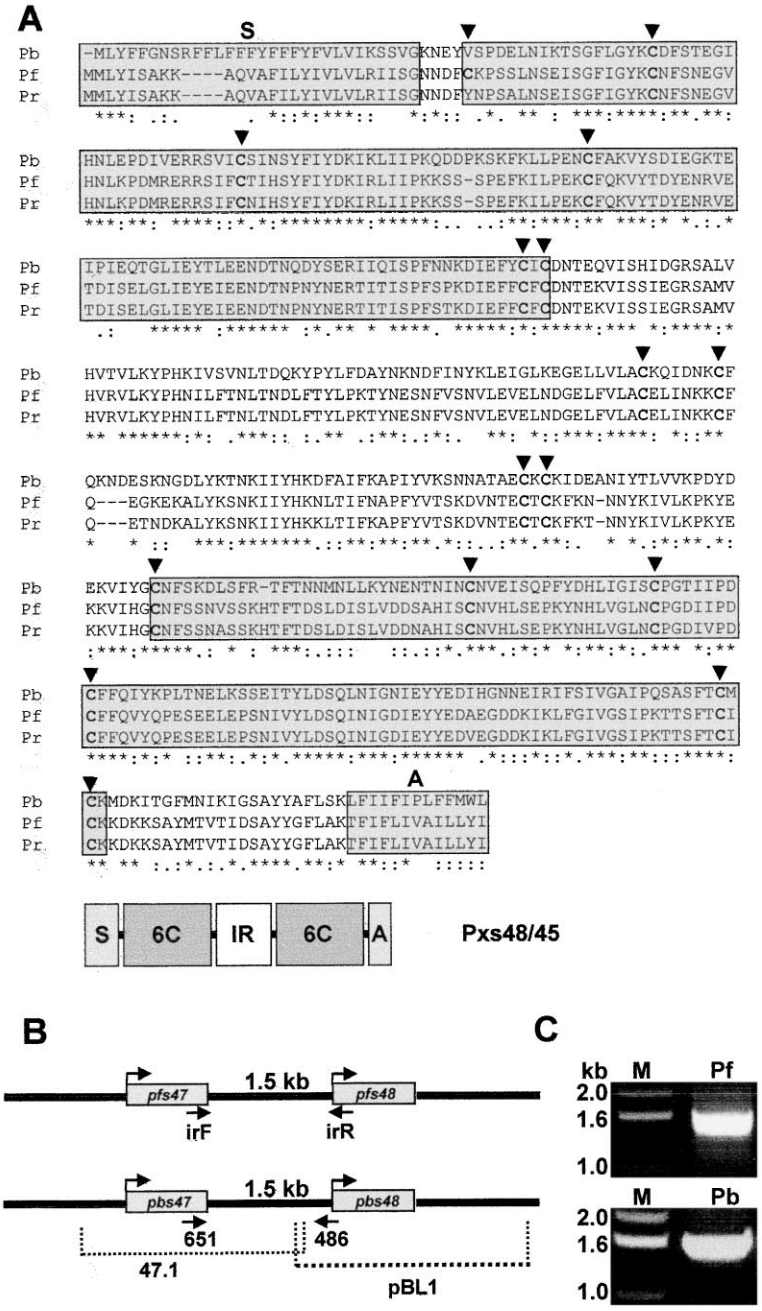

Figure 1. Comparison of the P48/45 Proteins and Organization of Their Genetic Loci

(A) Comparison of the predicted amino acid sequence of the P48/45 protein of *P. berghei*, *P. falciparum*, and the nonhuman primate parasite *P. reichenowi.* The different domains, including the two six-cysteine domains, are shaded and schematically shown. Arrowheads indicate the conserved cysteine residues. *, identical; :, conserved; ., semi-conserved substitution. S, secretory signal sequence; 6C, six-cysteine domain; IR, intervening region; A, anchor domain.

(B) Schematic diagram of the P48/45 and P47 genome organization in *P. falciparum* and *P. berghei*. Overlapping *P. berghei* genomic clones, 47.1 and pBL1, and the position of oligonucleotide primers used to amplify the intergenic regions are shown.

(C) PCR analysis demonstrating linkage of the P48/45 and the P47 gene in the genome of *P. falciparum* (Pf) and *P. berghei* (Pb).

Results

Identification of a Pfs48/45 Ortholog in *P. berghei*

pbs48/45 was isolated from a *P. berghei* genomic DNA library. Pfs48/45 and Pbs48/45 are 71% and 54% identical at the DNA and protein level, respectively (Figure 1A). All cysteine residues are positionally conserved apart from a single cysteine residue that is also not coded in the Prs48/45 gene of *P. reichenowi*, a chimpanzee parasite closely related to *P. falciparum* (Figure 1A). *pbs48/45* is located on one of the two largest chromosomes of *P. berghei* (chromosome 13/14; Figure 2B). Transcription of *pbs48/45* was in young (24 hr) and mature (30 hr) gametocytes (Figure 2C) and comparable to *pfs48/45*. Western analysis of gametocytes using specific polyclonal antiserum revealed a (broad) band of Pbs48/45 of 53 kDa, most probably consisting of a protein doublet that under reducing conditions migrated as a single well-resolved 55 kDa protein comparable to Pfs48/45 (Figure 2D).

P. berghei also Contains an Ortholog of *pf47*, Linked to *pbs48/45* in the Genome

BLAST analysis of the Pfs48/45 protein sequence against the *P. falciparum* genome sequence database showed that the 6-cys domain of Pfs48/45 aligns contiguously and most closely with Pf47, a gene described by Templeton and Kaslow (1999) (identity 26%, similarity

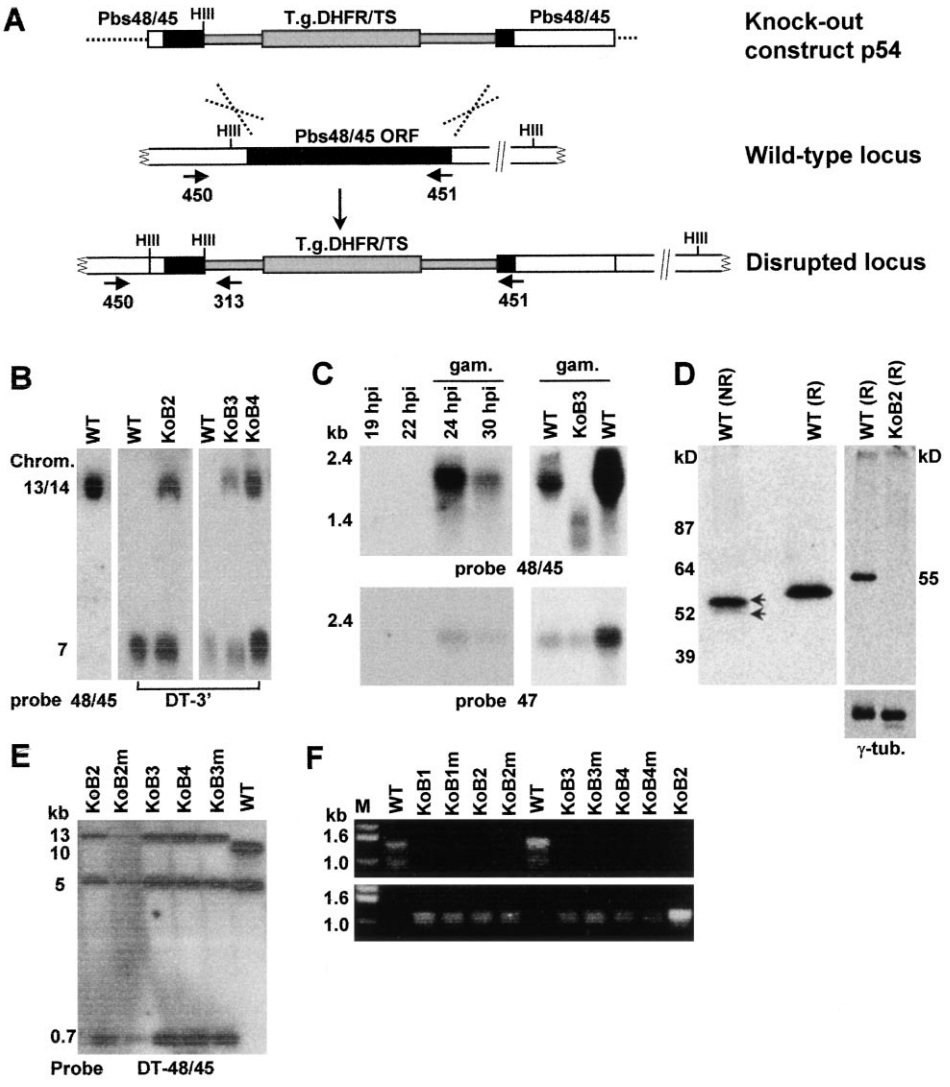

Figure 2. Generation of Pbs48/45ko Parasites of *P. berghei* and Analysis of the Genotype and Expression of Pbs48/45

(A) Schematic diagram of the *pbs48/45*-locus and the replacement vector p54 used to disrupt the Pbs48/45 gene. This vector contains the TgDHFR/TS selection cassette flanked by *pbs48/45* targeting sequences for replacement. Correct integration of the BamHI/KpnI fragment of p54 results in the disrupted gene as shown. Open box, *pbs48/45* untranslated regions; black box, *pbs48/45* coding region; hatched box, *T. gondii* DHFR/TS selection cassette; HIII, HindIII.

(B) Chromosome analysis of *P. berghei* wt and Pbs48/45ko parasites. Chromosomes were hybridized to a *pbs48/45*-specific probe (48/45; first panel) and to a probe specific for the *P. berghei dhfr/ts* 3′ UTR region (DT-3′; second and third panel). This latter probe hybridizes to the *dhfr/ts* locus on chromosome 7 and the *pbs45/48* locus in the Pbs48/45ko parasites as a result of integration of the selection cassette.

(C) Transcription of the Pbs48/45 and Pbs47 genes during blood stage development in wt and Pbs48/45ko parasites. The transcription pattern of the Pbs48/45 and Pbs47 genes in the wt parasite clone is shown in the left hand panel. Total RNA isolated from old trophozoites/young schizonts (16, 19, and 22 hr post invasion (hpi)) and from purified young (24 hpi) and mature (30 hpi) gametocytes (gam.) was subsequently hybridized to a *pbs48/45* locus- (48/45) and a *pbs47* locus- (47) specific probe. In the right hand panel, transcription of the Pbs48/45 and Pbs47 genes in young gametocytes (24 hpi) of wt and Pbs48/45ko parasite clones is shown.

(D) Western blot analysis of expression of Pbs48/45 in gametocytes of wt and ko parasites. Proteins were reacted with a polyclonal antiserum raised against a recombinant Pbs48/45 protein. A polyclonal antiserum against *P. falciparum* γ-tubulin was used as a control for the amount of protein loaded. NR, nonreduced; R, reduced.

(E) Southern blot analysis of HindIII-digested genomic DNA from wt and ko parasites demonstrates the expected disruption of the Pbs48/45 gene. DNA was hybridized to the probe DT-48/45, which detects the *dhfr/ts* locus as well as the *pbs48/45* locus. In wt parasites, a 5 kb fragment containing the *dhfr/ts* locus and a 10 kb fragment derived from the *pbs48/45* locus hybridized. In ko parasites, the 10 kb fragment increased in size to 13 kb and an additional fragment of 0.7 kb derived from the *pbs48/45* locus was obtained as a result of the integration event. The lanes KoB2m and KoB3m contain DNA collected from blood stages after mosquito (m) transmission of the Pbs48/45ko parasites KoB2 and KoB3.

(F) PCR analysis of genomic DNA of wt and ko parasites demonstrates correct disruption of *pbs48/45*. Using primers #450/#451 that specifically amplify the wt Pbs48/45 gene showed a PCR fragment of 1.5 kb in wt parasites (upper panel). Integration-specific PCR primers #450/#313 amplified the expected fragment of 1.3 kb only in the Pbs48/45ko parasites. The lanes KoB1–4m contain fragments amplified from DNA collected from blood stages after mosquito (m) transmission of the ko parasites.

43%). It has been found before that two genes expressed during sexual development, *pfs230* and *pfs25*, are closely linked to paralogous genes in the *Plasmodium* genome (Duffy and Kaslow, 1997; Gardner et al., 1998). We therefore examined the possible linkage of the Pfs48/45 and Pf47 genes. Assembly of sequence data of *pfs48/45* and *pf47* obtained from early release of sequences from the *P. falciparum* genome sequencing projects indicated that *pf47* is closely linked to *pfs48/45* (Figure 1B) and lies only 1.5 kb immediately upstream of *pfs48/45* (Figure 1C).

We identified a potential ortholog of *pf47*, *pb47*, as a sequence tag in the *P. berghei* gene sequence tag project (clone UFL_258PbD10, see Experimental Procedures) and used it as a probe to isolate the complete Pb47 gene whose ORF has an identity of 50% with Pfs47 and with Pfs48/45 of only 27%. All the cysteine residues within the 6-cys domains of Pb47 were conserved. *pb47* lies approximately 1.5 kb upstream of *pbs48/45* (Figure 1C) and is also transcribed in gametocytes (Figure 2C). Therefore, the genomic organization of the P48/45 and P47 genes is conserved in *P. falciparum* and *P. berghei* and they are a third paralogous gene pair expressed during sexual development.

Generation of P48/45ko Parasites

To study the function of the P48/45 proteins, P48/45ko parasites of *P. berghei* and *P. falciparum* were generated. The *P. berghei* Pbs48/45 gene was disrupted by replacement using a linear fragment of plasmid p54 (Figure 2A). Four Pbs48/45ko clones from two independent experiments, KoB1–4, were analyzed further by PCR and Southern blot analyses of separated chromosomes and restriction fragments (Figures 2B, 2E, and 2F) to confirm the expected disruption of the wild-type (wt) *pbs48/45* locus.

Pbs48/45ko parasites produced normal numbers of female and male gametocytes (see below). Northern analysis of total RNA and Western analysis of proteins obtained from purified gametocytes of the Pbs48/45ko parasites demonstrated that the disrupted Pbs48/45 gene was not transcribed and the protein was absent (Figures 2C and 2D). The level of transcription of the paralog *pbs47* in Pbs48/45ko parasites was unaffected (Figure 2C). Therefore, phenotypic effects observed in Pbs48/45ko parasites are solely due to a lack of Pbs48/45 in this mutant.

The *P. falciparum* Pfs48/45 gene was disrupted using plasmid pI48 that generates two nonfunctional copies of *pfs48/45* in the *P. falciparum* genome following site-specific integration of pI48 (Figure 3A). In contrast to the double crossover mechanism of site-specific integration of p54 in *P. berghei*, the single crossover mechanism of integration used in *P. falciparum* is potentially a reversible event. Reversion would lead to the regeneration of the wt gene in parasites as has been described for *P. berghei* (Ménard and Janse, 1997). In two independent experiments, we transfected *P. falciparum* parasites and obtained clones that were both pyrimethamine-resistant and contained a disrupted Pfs48/45 gene. Two clones from each experiment, KoF1–4, were chosen for further analysis. PCR, Southern blot, and plasmid rescue analyses of Pfs48/45ko parasite DNA demonstrated that these parasites contain pI48 correctly integrated at the *pfs48/45*-locus (some in a concatemeric arrangement), but also episomally maintained plasmids (Figures 3B and 3C).

No Pfs48/45 protein could be detected in most of the Pfs48/45ko clones by immunofluorescence assay (IFA) and Western blot analysis of proteins from purified gametocytes (Figures 3D and 3E) whereas in wt parasites, all mature (male and female) gametocytes reacted strongly by IFA (Figure 3D). The level of transcription of the paralog Pf47 and a control gene *pfs16* was comparable in Pfs48/45ko and in wt parasites as determined by quantitative PCR (data not shown). In some of the Pfs48/45ko clones, however, we could detect by PCR low levels of parasites with the wt genotype, even in multiply cloned lines. Some pfs48/45ko gametocytes of the clones (*f* 10^{-4}–10^{-6}) reacted strongly with anti-Pfs48/45 antibodies by IFA. Phenotype analysis of Pfs48/45ko parasites may, therefore, be complicated by low frequency reversion to the wild type. Pfs230 has been shown to form a complex with Pfs48/45 at the gametocyte/gamete surface (Kumar, 1987). Western blot analysis using mAb 18F25 specific for Pfs230 demonstrated that the expression and processing (Brooks and Williamson, 2000) of Pfs230 was unaffected in Pfs48/45ko parasites (Figure 3E and data not shown).

P48/45ko Parasites Produce Normal Numbers of Gametocytes and Gametes

As expected, disruption of the P48/45 gene had no observable effect on blood stage asexual development. In *P. berghei*, the multiplication rate of asexual parasites in vivo, the duration of the asexual cycle, and the number of merozoites in mature schizonts did not differ between Pbs48/45ko and wt parasites. In *P. falciparum*, Pfs48/45ko parasites showed a normal growth and multiplication rate in 14-day cultures (data not shown). The disruption of P48/45 did not affect the production or ratio of male and female gametocytes in either *P. berghei* or *P. falciparum* (Tables 1 and 2). Furthermore, every characteristic of the development of P48/45ko gametocytes into gametes was comparable to wt; conversion rate, morphology, male gamete motility, and their attachment to uninfected erythrocytes, resulting in the presence of characteristic exflagellation centers, female gamete emergence, and their expression of the gamete/zygote-specific protein Pbs21 or Pfs25 on their surfaces, respectively (Table 2; Figure 4B).

Zygote Formation and Transmission Capacity Is Strongly Impaired in P48/45ko Parasites

Although disruption of the P48/45 gene did not have an observable effect on gametocyte and gamete development, there was a dramatic effect on development of both *P. falciparum* and *P. berghei* zygotes and ookinetes. In vitro, 52%–69% of the wt *P. berghei* female gametes compared with only 0.001%–0.03% of Pbs48/45ko female gametes developed into mature ookinetes (Table 2). In *P. falciparum,* ookinetes were readily observed in midguts of mosquitoes infected with wt parasites whereas no ookinetes were found in mosquitoes infected with Pfs48/45ko parasites (Table 1).

To test whether the observed inhibition of ookinete

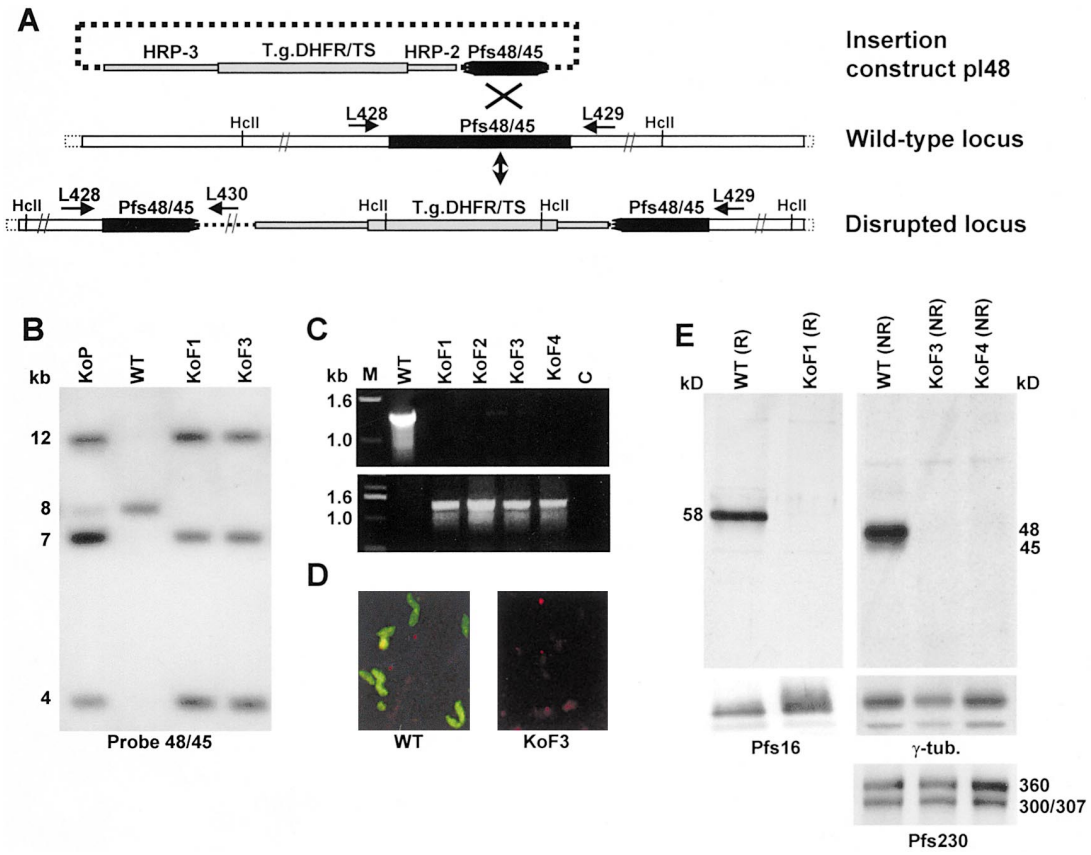

Figure 3. Generation of Pfs48/45ko Parasites (clone KoF1-KoF4) of *P. falciparum* and Analysis of the Genotype and Expression of Pfs48/45

(A) Schematic diagram of the *pfs48/45*-locus and insertion vector pl48 used to disrupt the Pfs48/45 gene. This vector contains a TgDHFR/TS selection cassette and a truncated copy of the Pfs48/45 gene, which serves as the site for homologous recombination. Correct site-specific integration of this plasmid results in the disrupted gene as shown. Open box, genomic DNA; black box, *pfs48/45* ORF; hatched box, *T. gondii* DHFR/TS selection cassette; dotted line, plasmid sequences; HcII, HincII.

(B) Southern blot analysis of HincII-digested genomic DNA from wt and Pfs48/45ko parasites demonstrates the expected disruption of Pfs48/45 gene. DNA was hybridized to the *pfs48/45*-specific probe. In wt parasites, this probe hybridized to a single fragment of 8 kb, whereas it hybridizes to three fragments in Pfs48/45ko parasites. Two fragments of 4 kb and 12 kb arise as a result from correct integration of the insertion plasmid in the Pfs48/45 gene, whereas the third band of 7.3 kb corresponds to either the presence of episomally maintained plasmids or the existence of multiple copies of the insertion plasmid in the genome. In the Pfs48/45ko clones, no hybridization was obtained with the 8 kb fragment of the wt gene. The presence of episomes was confirmed by performing plasmid rescue experiments (data not shown). KoP, parental Pfs48/45ko parasite population.

(C) PCR analysis of genomic DNA of wt and Pfs48/45ko parasites demonstrates correct disruption of Pfs45/48 gene in the Pfs48/45ko parasites. Primers #428/#429 specifically amplify a 1.2 kb fragment of the wt Pfs48/45 gene (upper panel). Integration-specific primers #428 and #430 amplified the recombinant 1.3 kb fragment only in the Pfs48/45ko parasites (lower panel).

(D) Expression of Pfs48/45 in gametocytes of wt and Pfs48/45ko parasites as shown in an IFA using FITC-conjugated mAb 32F3. Pfs48/45 is present in both male and female wt gametocytes and absent in gametocytes of the Pfs48/45ko (KoF3) clone.

(E) Western blot analysis of protein expression in gametocytes of wt and Pfs48/45ko parasites. Proteins were reacted with the following polyclonal antisera: anti-Pfs48/45 (K96); anti-Pfs16, a *P. falciparum* gametocyte protein; *P. falciparum* γ-tubulin and mAb 18F25 specific for Pfs230. R, reduced; NR, nonreduced.

formation in the P48/45ko parasites correlated with a reduction in transmission capacity, as defined by the rate of oocyst production, mosquitoes were fed with Pfs48/45ko and Pbs48/45ko gametocytes. P48/45ko oocyst production was strongly reduced compared to oocyst production in wild-type parasites (Tables 1 and 2). In *P. falciparum,* a maximum of 2.5% of the mosquitoes were infected (max. one oocyst per mosquito) compared to an average wt infection rate of 93% (max. 180 oocysts per mosquito). It is possible that these oocysts resulted from fertilization of gametes that have reverted to wt.

Pbs48/45ko oocyst numbers are 27–63 times lower compared to wt parasites (Table 2). In the mosquito midgut, an average of 0.09% of Pbs48/45ko female gametes developed into ookinetes, which is significantly lower than in wt parasites (average of 9.2%) but is higher than the average in vitro conversion rate of 0.02%. Therefore, Pbs48/45ko parasites were able to produce oocysts at a significantly higher rate than expected on the basis of inhibition of ookinete production in vitro. The oocysts had normal morphology under light microscopy and produced sporozoites 10–12 days after mosquito infections. Pbs48/45ko sporozoites were able to infect naïve mice as effectively as wt *P. berghei* sporozoites (data not shown). Transmitted Pbs48/45ko parasites

Table 1. Gamete Formation in Pfs48/45ko Parasites Is Normal but Ookinete and Oocyst Production Is Strongly Inhibited

Parasite	Gametocyte Production[a]	Sex Ratio ♂ : ♀	Ookinete Production[b]	Oocyst Production[c]	Infected Mosquitoes (%)
Wild-type (wt)	0.5	1:2.5	272	98	93
Range; n = 8	(0.3–1.0)	(0.7–3.5)	(39–566)	(20–180)	(78–100)
KoF1	0.5	1:2.5	0	0.007	0.5
Range; n = 12	(0.1–0.8)	(1.4–4.4)		(0–0.03)	(0–2.5)
KoF2	0.7	1:2.2	0	0.0005	0.35
Range; n = 2	(0.2–1.2)	(1.4–3)		(0–0.001)	(0–0.7)
KoF3	1.3	1:2.7	0	0.004	0.3
Range; n = 5	(0.4–2.0)	(1–4)		(0–0.01)	(0–1)

[a] Gametocyte production is the number of mature gametocytes per 10^2 erythrocytes at day 14 after start of the cultures and the pfs48/45ko parasites (KoF1–3) are comparable to wild type.
[b] Ookinete production is the mean number of ookinetes per mosquito midgut 21 hr after feeding.
[c] Oocyst production is the mean number of oocysts at day 7 after feeding mosquitoes.

maintained the disrupted *pbs48/45* genotype (Figure 2F) and the observed reduction in the rate of ookinete and oocyst production of Pbs48/45ko parasites (data not shown).

Pbs48/45ko Male Gametes Have an Impaired Ability to Attach to and Penetrate Female Gametes

The reduced capacity of P48/45ko gametes to develop into zygotes suggests that P48/45 plays a role in fertilization or subsequent zygote development. The role of Pbs48/45 in *P. berghei* was examined using in vitro fertilization assays. The behavior of gametes was examined in vitro after induction of gamete development. In six independent preparations of wt parasites, motile male gametes were observed attaching to erythrocytes and to female gametes. Individual fertilization events involving male penetration of a female gamete were readily observed (a range of 6–18 fertilization events per preparation). In contrast, in ten independent preparations of Pbs48/45ko parasites (clone KoB2 and KoB3), no single fertilization event was detected. Motile Pbs48/45ko male gametes attached only to erythrocytes and not to female gametes. The observed fertilization defect was confirmed by examination of Giemsa-stained parasites prepared 1 hr after induction of gamete formation. In two independent preparations of wt parasites, 46% and 54% of the female gametes had been fertilized and exhibited the characteristic features of a zygote (Janse et al., 1985a; 1986); 15% and 21% of these zygotes contained the two, still separated, nuclei of the female and male gametes. These fertilization features were absent from equivalent preparations of Pbs48/45ko parasites. Only large clusters of single nucleated female gametes were found (Figure 4A). Therefore, fertilization is strongly impaired in Pbs48/45ko parasites.

Gamete Fertility Is Strongly Impaired in Pbs48/45ko Male but Not Female Gametes

P48/45 is present on the surface of both male and female gametes. The fertilization defect of Pbs48/45ko gametes could result, therefore, from a loss of either male or female fertility, or of both. To discriminate between these possibilities, we performed in vitro cross-fertilization experiments between gametes of different *P. berghei* wt and Pbs48/45ko clones.

To test the fertility of Pbs48/45ko male gametes, the conversion rate of wt female gametes into ookinetes was determined after mixing Pbs48/45ko male and female gametes with wt female gametes. Formation of wt male gametes was blocked by the DNA polymerase I inhibitor Aphidicolin. Three independent crosses produced no ookinetes (Table 3; cross B), indicating that Pbs48/45ko male gametes were unable to fertilize wt female gametes. To prove the viability of the wt female gametes in cross B after exposure to Aphidicolin, wt female gametes were mixed with male and female gametes of the Pbs21ko clone. This clone produces normal numbers of gametes and ookinetes that lack the Pbs21 protein on the surface (A. Tomas et al., submitted). The conversion rate of wt female gametes into ookinetes was determined by counting the number of ookinetes expressing Pbs21 visualized with a specific mAb. Large numbers of ookinetes were Pbs21 positive (Table 3; cross A), demonstrating that wt female gametes could be fertilized by the Pbs21ko male gametes. Pbs21-negative ookinetes were also present resulting from normal self-fertilization of the Pbs21ko parasites. Therefore, wt female gamete fertility is unaffected after Aphidicolin treatment (cross A) and male Pbs48/45ko gametes are infertile (cross B).

Since the P48/45 protein is abundantly present on the surface of wt female gametes, we also expected females to contribute to the lack of self-fertilization of P48/45ko parasites. To investigate the fertility of the Pbs48/45ko female gametes, we again mixed gametes of the Pbs48/45ko parasites with gametes of the Pbs21ko clone and counted Pbs21-positive and -negative ookinetes (Table 3; cross C). If Pbs48/45ko females were infertile, only Pbs21-negative ookinetes from the self-fertilization of Pbs21ko mutant gametes would be observed. Unexpectedly, in five independent repetitions of this assay, we observed ookinetes that reacted strongly with the anti-Pbs21 antibody, which can only result from fertilization of Pbs48/45ko female gametes by Pbs21ko males (Table 3, cross C; Figure 4B). The conversion rate of the Pbs48/45ko female gametes into ookinetes was comparable to the conversion rates obtained in standard fertilization assays of wt parasites (Table 3, cross C). Therefore, disruption of Pbs48/45 does not affect fertility of female gametes or further development of zygotes.

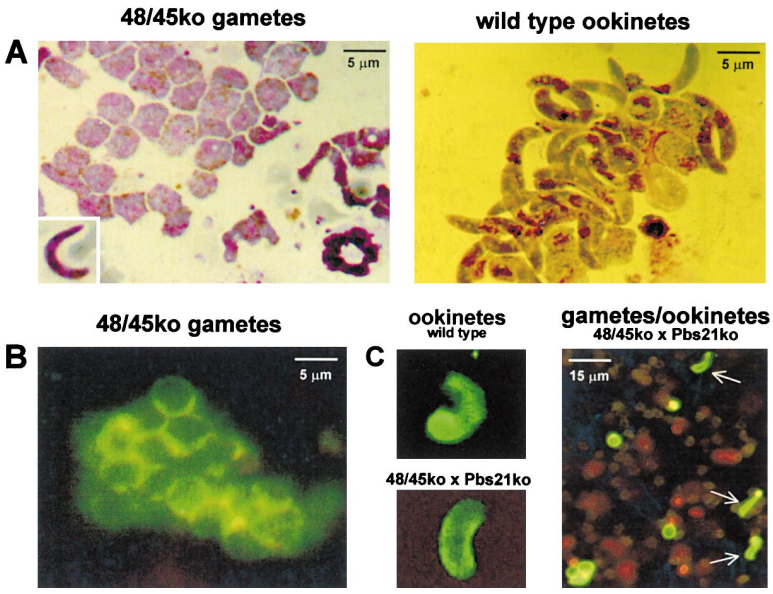

Figure 4. Female Gametes and Ookinetes of Pbs48/45ko Parasites Produced during In Vitro Fertilization Assays

(A) Large clusters of unfertilized female gametes are present in Pbs48/45ko parasites 16 hr after induction of gamete formation, whereas in wt parasites characteristic clusters of ookinetes are observed. Parasites shown are stained with Giemsa and photographed at 1000× magnification. One of the few ookinetes found in the in vitro cultures of Pbs48/45ko parasites is shown in the insert.

(B) Unfertilized female gametes of Pbs48/45ko parasites express the Pbs21 surface protein specific for female gametes and ookinetes. Parasites are stained with mAb 13.1 and photographed at 1000× magnification.

(C) Ookinetes of Pbs48/45ko parasites, resulting from cross-fertilization of the Pbs21-positive female gametes of Pbs48/45ko parasites by males from the Pbs21ko parasite clone 602. Parasites visualized as in (B). Round forms are unfertilized female gametes and arrows indicate mature ookinetes.

Discussion

P48/45 Is Necessary for Male Gamete Fertility in *Plasmodium*

Disruption of P48/45 strongly reduces zygote formation in both species resulting from a dramatic diminution in male gamete fertility. In addition, although P48/45 is expressed abundantly on the surface of female gametes (Kaushal and Carter, 1984; Vermeulen et al., 1985), it has no apparent effect on the ability of female gametes to be formed or fertilized. The inability of P48/45ko male gametes to adhere to and penetrate female gametes indicates that P48/45 plays a direct role in fertilization mediated by male gametes, which may include recognition, adhesion, or presentation of accessory molecules such as P230. In *P. falciparum*, male gametes attach to erythrocytes in a sialic acid and glycophorin-dependent manner (Templeton et al., 1998). Disruption of *pbs48/45* does not affect this interaction, however, suggesting that P48/45 is not the gamete receptor for erythrocyte surface molecules.

Ko parasites defective in male gamete fertility will be useful tools for the identification and study of additional genes involved in the fertilization process of malaria parasites. Other mutants of *Plasmodium* have been described that show aberrant sexual development that remains undescribed at the molecular level (Guinet et al., 1996). Genetic studies have now started to define

Table 2. Gamete Formation in Pbs48/45ko Parasites Is Normal but Ookinete and Oocyst Production Is Strongly Inhibited

Parasite	CR (vivo) Asexual/ Gametocyte[a]	Sex Ratio ♂ : ♀	CR (vitro) ♀ Gam/ ♀ Gamete[b]	CR (vitro) ♂ Gam/ ♂ Gamete[c]	CR ♀ Gam/Ookinete In Vitro[d]	CR ♀ Gam/Ookinete In Vivo[e]	Oocysts Production[f]	Infected Mosquitoes (%)
wt mean	19	1:1.7	90	77	64	9.2	379	97
(range)	(16–23)	(1.4–2.3)	(86–93)	(72–81)	(52–69)	(4.2–16.8)	(146–983)	(90–100)
	n = 6	n = 5	n = 6	n = 6	n = 6	n = 8	n = 8	n = 8
KoB2 mean	17	1:1.3	88	73	0.02		14	69
(range)	(15–18)	(1.2–1.4)	(86–90)	(70–76)	(0.001–0.03)	ND	(0.7–27)	(45–93)
	n = 2	n = 2	n = 2	n = 2	n = 2		n = 2	n = 2
KoB3 mean	19	1:1.6	85	70	0.01	0.09	6	52
(range)	(21–16)	(1.5–1.6)	(81–91)	(68–73)	(0.005–0.02)	(0.03–0.16)	(0–34)	(23–80)
	n = 2	n = 2	n = 2	n = 2	n = 2	n = 2	n = 2	n = 2
KoB4 mean	16	1:1.8	96	75			19	69
(range)	(15–16)	(1.7–1.9)	(95–96)	(73–78)	ND	ND	1–34	35–95
	n = 2	n = 2	n = 2	n = 2			n = 5	n = 5

[a] Conversion (CR) rate of asexual parasites into gametocytes is the percentage of ring forms that develop into gametocytes in synchronized infections in mice and wild-type (wt) and Pbs84/54ko (KoB2–4) parasites are comparable.

[b] Conversion rate of gametocytes into ♀ gametes is the percentage of ♀ gametocytes that escape from the host cell and subsequently express the Pbs21 protein on their surface in in vitro cultures.

[c] Conversion rate of ♂ gametocytes into ♂ gametes is the percentage of ♂ gametocytes that underwent exflagellation in in vitro cultures.

[d] Conversion rate of female gametes into ookinetes in vitro is the percentage of female gametes that develop within 18 hr into ookinetes in in vitro cultures.

[e] Conversion rate of female gametes into ookinetes in vivo is the percentage of Pbs21-positive ookinetes of the total of Pbs21-positive cells (gametes and ookinetes) present in the midguts of mosquitoes 21 hr after feeding.

[f] Oocyst production is the mean number of oocysts at day 7 after feeding mosquitoes on infected mice.

Table 3. Cross Fertilization Studies Reveal that Male Gametes of Pbs48/45ko Parasites Are Infertile yet Female Gametes Can Be Fertilized

Parasite	Aphi.[d]	♂ Gam[e]	♀ Gam[e] Pbs21 +/−	Ookinetes[e] Pbs21 +/−	Conversion Rate ♀ Gam/Ookinete
Controls					
wt	−	590	590+	203+	34
wt	+	0	590+	0	0
Pbs21ko	−	365	365−	150−	41
Pbs48/45ko	−	558	558+	0	0
Cross A[af]					
wt x	+	0	197+	52+	26
Pbs21ko	−	243	243−	89−	37
Cross B[bf]					
wt x	+	0	295+	0	0
Pbs48/45ko	−	279	279+	0	0
Cross C[cf]					
Pbs21ko x	−	243	243−	81−	33
Pbs48/45ko	−	186	186+	84+	45

[a] Cross A: Male gametes of Pbs21ko parasites were able to fertilize female gametes (Pbs21-positive) of wt parasites.
[b] Cross B: Male gametes of Pbs45/48ko (KoB4) parasites were unable to fertilize female gametes of wt parasites.
[c] Cross C: Pbs21-positive female gametes of Pbs48/45ko (KoB4) parasites were fertilized by male gametes of Pbs21ko parasites. The outcome of this cross was unaffected by prior treatment of Pbs48/45ko (KoB4) gametes with 5×10^{-4} M Aphidicolin.
[d] Male gamete formation in the wt parasites was blocked by 5×10^{-4} M Aphidicolin (Aphi).
[e] The numbers of gametocytes and ookinetes are expressed per 10^5 erythrocytes. The number of male gametocytes is determined by counting the number of exflagellations. Male/female ratio in infected blood of the different clones was approximately 1 (i.e., the number of female gametocytes equals the number of males).
[f] In the crosses, we mixed infected blood in amounts based on the numbers of gametocytes present in the infected blood: Cross A: 4 μl wt and: 8 μl Pbs21ko; Cross B: 6 μl wt and 6 μl Pbs48/45ko; Cross C: 8 μl Pbs21ko and 4 μl Pbs48/45ko. Inhibition of male gamete formation in wt parasites was achieved by adding Aphidicolin to the culture medium at a concentration of 5×10^{-4} M during the first 10 min, after which Aphidicolin was removed by washing. Gametes of the different clones were then mixed in fresh culture medium and incubated for 18 hr. Comparable conversion rates for Pbs48/45ko (KoB4) female gametes into ookinetes were observed in the absence (26%) of presence (27%) of drug. Pbs21ko female gametes and ookinetes can be distinguished from the gametes/ookinetes of wt and Pbs48/45ko parasites by the lack of staining with mAb 13.1 (Pbs21-positive parasites).

the role of sex-specific proteins. Disruption of the gene encoding Pfg27 through genetic modification resulted in the arrest of early gametocyte development (Lobo et al., 1999), and a genetic cross of *P. falciparum* demonstrated that an as yet unidentified gene residing on chromosome 12 plays a role in male fertility (Vaidya et al., 1995). Identification of proteins involved in the sexual development and determination of their function might facilitate design of improved strategies for blocking transmission of malaria parasites.

Members of the 6-Cys Domain Gene Superfamily Are Unique and Conserved within the *Plasmodium* Genus and Expressed during Sexual Development

In general, the mutual recognition by male and female gametes required for fertilization is mediated by structurally diverse sex- and species-specific gamete surface receptor proteins. These proteins are typically rapidly evolving, highly structured members of families of protein receptors that participate in specific protein–protein interactions leading to and ensuring sex specificity of fertilization (Vacquier, 1998). P48/45 belongs to a family of proteins that share a conserved structure defined by apparently species-specific 6-cys domains (Carter et al., 1995; Templeton and Kaslow, 1999). Database searches do not reveal any Pfs48/45 or other 6-cys domain family member homologs outside the genus *Plasmodium*, including the related Apicomplexan *Toxoplasma gondii.* Although full genome sequences are necessary, current indications are that the 6-cys motif is unique to and distributed throughout *Plasmodium*. Most members of the Pfs48/45 superfamily are conserved in the genome of other human malarias (*P. vivax*) and the rodent parasites, *P. berghei* and *P. chabaudi*.

A further 6-cys superfamily member, P47, is also expressed during sexual development in both *P. falciparum* and *P. berghei* and is predicted to be a gametocyte/gamete-surface antigen. All members of the 6-cys domain superfamily characterized to date are conserved and are gametocyte/gamete-specific suggesting that this family has evolved to play a role in the sexual cycle of *Plasmodium.* Whether all these proteins mediate gamete interactions, as exemplified by P48/45, remains to be determined. Interestingly, it has been shown that Pfs48/45 physically interacts with another member of the protein family, Pfs230, and antibodies to Pfs230 can also inhibit the development of zygotes (Kumar, 1987; Williamson et al., 1993; Kaslow, 1996).

Linkage of *p48/45* and its paralog, *p47*, is conserved in the genome of *P. berghei* and *P. falciparum.* Tight linkage of closely related genes that are specifically transcribed in gametocytes and encode surface proteins appears to be a recurrent theme in malaria parasites. The genes encoding Pfs230 and P25, transcribed during the sexual stages, form tightly linked gene pairs with their paralogs, separated by approximately 2 kb (Gardner et al., 1998; A. Tomas et al., submitted). Gene duplication resulting in paralogous gene pairs appears, therefore, to be an ancient and coordinated event in *Plasmodium*. We have recently demonstrated a high level of redundancy between the P25 gene pair (A. Tomas et al., submitted). Knockout of either gene encoding

those proteins does not result in a significant inhibition of zygote and oocyst development, whereas the development of zygotes into oocysts is blocked when both genes are disrupted. In contrast, this study shows that P47 cannot compensate in the same way for P48/45 disruption. Furthermore, the few P48/45ko parasites that can be transmitted by mosquitoes still exhibit the defective male gamete phenotype after mosquito transmission. The possibility is, therefore, excluded that a proportion of the parasites stably switch to the expression of alternative genes that are able to complement the defect of disruption of P48/45. Stable switching of genotype and phenotype has been shown before in *Plasmodium* in relation to switching antigenic types (al-Khedery et al., 1999; Wahlgren et al., 1999) and pathways of erythrocyte invasion (Dolan et al., 1990; Reed et al., 2000).

Implications for Vaccine Development

This study demonstrates the important role of Pfs48/45 in malaria parasite transmission and emphasizes its value as a leading candidate antigen for the development of a transmission-blocking vaccine. However, disruption of the P48/45 gene did not result in complete blockage of transmission. Malaria transmission is remarkably efficient even at low gamete densities (Janse et al., 1985b; Boudin et al., 1993), and it is likely that factors present in the mosquito midgut can enhance fertilization (Billker et al., 1998). These observations are reinforced by the demonstration here that, although P48/45ko zygote and ookinete formation is almost undetectable in vitro, P48/45ko parasites do undergo low-level transmission to the mosquito. These findings might have serious implications for development of transmission-blocking vaccines and highlight the need to identify additional molecules for the formulation of a multivalent vaccine that completely blocks transmission and guards against the selection of escape mutants. We also found that reduction in zygote formation in vivo is less marked in Pbs48/45ko parasites than in Pfs48/45ko parasites. The assays used to quantify zygote formation differ between the two parasites and these distinctions may explain the apparently more efficient zygote formation in ko *P. berghei* parasites. Transmission of in vitro cultivated gametocytes to mosquitoes via membrane feeding is significantly less efficient than direct mosquito feeding on an infected host (C. J., unpublished observations). It is not possible to perform human transmission studies of *P. falciparum* ko parasites and so *P. berghei* is a useful and more sensitive model system to test the essential nature of transmission-blocking candidate antigens.

Members of the 6-cys domain gene superfamily possess important features that make them attractive targets for a transmission-blocking vaccine: (1) There is remarkable conservation of the different p48/45 superfamily members within *Plasmodium*; (2) this conservation and genus-wide distribution indicates that the molecular mechanism of fertilization and the individual roles of this family of proteins are also highly conserved; and (3) most importantly, it appears that a number of these proteins are already expressed on the surface of the gametocytes in the blood, and therefore, it is possible that immune responses against these proteins will be boosted by natural infection. Antibodies against Pfs48/45 and Pfs230 are present in naturally infected persons and serum levels of Pfs48/45 antibodies correlate with transmission-blocking activity (Graves et al., 1992; Roeffen et al., 1996; Mulder et al., 1999). Characterization of the pattern of expression and possible role of the other 6-cys family members in gamete development and fertilization is, therefore, desirable. This and an evaluation of the immunological properties of the 6-cys domain gene superfamily members will assist in a rational approach to transmission-blocking vaccine design.

Experimental Procedures

Parasites

P. berghei: Clone 15cy1A (ANKA), a gametocyte-producer clone, used as the reference parasite; Clone 233 (ANKA), a nongametocyte-producer clone (Janse et al., 1989); Clone 602, a knockout mutant parasite lacking the Pbs21 surface protein of gametes/zygotes (A. Tomas et al., submitted). *P. falciparum:* Cloned line NF54.

Handling of *P. berghei* was as described (Janse and Waters, 1995). *P. falciparum* parasites were cultured using a semi-automated culture system. Asexual parasites were removed by treatment of the cultures with 50 mM N-acetyl-glucosamine between days 8 and 12 (Gupta et al., 1985). Mature gametocytes were isolated and concentrated as described by Staalsoe et al., 1999.

Isolation and Characterization of the Pbs48/45 and Pb47 Genes

A λZAP *P. berghei* genomic DNA library was screened using 1 kb of the Pfs48/45 gene as a probe, amplified by PCR using primers #343 and #344 (oligonucleotides used in this study are available at http://www.cell.com/cgi/content/full/104/1/153/DC1) using standard conditions (Birago et al., 1996). A clone, pBL1, containing the Pbs48/45 coding region, 358 bp of upstream and 1.5 kb of downstream region, was further characterized and sequenced. The Pb47 gene was cloned by gene walking upstream of Pbs48/45 and through use of clone from the University of Florida *P. berghei* genome sequencing tag project (http://parasite.vetmed.ufl.edu). A genomic clone, 47.1, containing the Pbs47 coding region, 500 bp of upstream and 1.3 kb of downstream region, was isolated and sequenced.

Analysis of *p48/45* and *p47* Linkage in the Genome

A contig of the Pfs48/45 and Pf47 locus was assembled from BLAST analysis of sequence data for *P. falciparum* chromosome 13 (http://www.sanger.ac.uk/Projects/P_falciparum/). Sequencing of *P. falciparum* chromosome 13 was accomplished as part of the Malaria Genome Project with support by The Wellcome Trust. Preliminary sequence data for *P. falciparum* chromosomes 10 and 11 were obtained from The Institute for Genomic Research (www.tigr.org). For *P. falciparum*, primer pair irR (nt 14–25) and irF (nt 1206–1227) of *pfs48/45* and *pf47*, respectively, and for *P. berghei*, primer pairs #486 and #651 corresponding to nt 1210–1233 of *pbs47* were used to amplify the intergenic region by PCR.

Generation of *P. falciparum* and *P. berghei* P48/45ko Parasites

P. berghei: The Pbs48/45 gene of *P. berghei* was disrupted with replacement vector p54, a derivative of the previously described construct $pD_B.D_{T\Delta H}.D_B$ (Wengelnik et al., 1999). The 5′ *pbs48/45* targeting sequence (0.7 kb) was amplified from clone pBL1 using primer #406 (200 nt upstream of the predicted start codon) introducing HindIII and BamHI sites, respectively, and primer #407 (nt 527–47) introducing an HindIII site, and cloned into $pD_B.D_{T\Delta H}.D_B$ giving rise to vector p53. The 3′ *pbs48/45* targeting sequence (1.5 kb) was amplified using primer #408 (nt 1219–39) introducing an EcoRV site in combination with the pBS-SK forward primer and cloned into p53 giving rise to the replacement construct p54 that was cut with BamHI and KpnI, the DNA fragment gel purified and used for transfection.

P. berghei parasites were transfected and cloned as described (Ménard and Janse, 1997).

P. falciparum: The Pfs48/45 gene of *P. falciparum* was disrupted with insertion vector pI48, a derivative of the previously described pDT.Tg23 (Wu et al., 1996). pI48 was constructed by cloning 1088 bp of the coding sequence of *pfs48/45* into pDT.Tg23. This fragment was obtained by PCR amplification using primers #343/#344 containing BglII and SpeI sites, respectively, and cloned into pDT.Tg23. Transfection of *P. falciparum* blood stage parasites with vector pI48, selection, and cloning of recombinant parasites were as described by Fidock and Wellems,1997. To select *pfs48/45*ko clones, the absence of expression of the Pfs48/45 in mature gametocytes was assayed by immunofluorescence assay (IFA).

Genotype Analysis of P48/45ko Parasites

Disruption of the Pbs48/45 and Pfs48/45 gene in the ko parasites was analyzed by PCR, DNA, and RNA blot analyses (Ménard and Janse, 1997). In *P. berghei* parasites, homologous integration at the target gene was detected by PCR amplification using a primer pair #450/#313 and #428/#430 for *P. falciparum*. Control PCR amplifications to detect the wt gene were performed using primer pairs, *P. berghei*, #450/#451; *P. falciparum*, #428/#429. *P. berghei* chromosomes were separated by Field Inversion Gel Electrophoresis (FIGE) as described (Birago et al., 1996) and hybridized to a *pbs48/45* ORF probe or a probe of the 3′UTR of the *P. berghei dhfr/ts* locus (DT-3′) (Wengelnik et al., 1999). *P. berghei* genomic DNA, digested with HindIII, was hybridized to a hybrid probe (DT-48/45, amplified using primers #450/#313) that hybridizes to the 5′UTR of the *P. berghei* DHFR/TS gene and the *pbs48/45* locus (see Figure 2A). Genomic DNA from *P. falciparum,* digested with HincII, was hybridized to a Pfs48/45 ORF-specific probe.

Generation of Pbs48/45-Specific Antiserum Using Recombinant Pbs48/45

A fragment of the Pbs48/45 gene (encoding amino acids 62–408) was amplified by PCR using primers pBEXP1and pBEXP2 and cloned into the expression vector pET-15b (Novagen) providing an N-terminal 6-Histidine tag under the control of the T7*lac* promoter (pET-pbs48/45). Recombinant Pbs48/45 were purified by affinity chromatography on Ni-NTA super flow beads (Qiagen) under denaturing conditions according to the manufacturer's instructions. Polyclonal antiserum was raised in a New Zealand rabbit by injection of 200 μg of gel-purified recombinant protein. Boosting was carried out subcutaneously with 3-week intervals using 200 μg recombinant protein in incomplete Freund's adjuvant. Serum (Pc48/45) obtained 2 weeks after the third boost was immunopurified on immobilized, purified recombinant Pbs48/45.

Immunofluorescence and Western Blotting

IFAs were performed on *P. falciparum* parasites air-dried on poly-l-lysine-coated multispot slides or on live gametes using Pfs48/45-specific mAb 32F3 (Roeffen et al., 1995). For Western blot analysis, total protein samples of purified *P. berghei* and *P. falciparum* gametocytes were fractionated on 10% SDS polyacrylamide gels in the presence or absence of 100 mM DTT. For *P. berghei* blots, Pc48/45 was used and rabbit polyclonal antibody against *P. falciparum* γ-tubulin as a control (Maessen et al., 1993). For *P. falciparum*, a Pfs48/45 rabbit polyclonal antiserum (K96) (Milek et al., 1998), a Pfs16 rabbit polyclonal antiserum (Moelans et al., 1995), and Pfs230-specific mAb 18F25 (Roeffen et al., 1995) were used. Antibody reactions were visualized by ECL detection.

Phenotype Analysis of P45/48ko Parasites

Transmission capacity of the Pfs48/45ko and Pbs48/45ko parasites was determined by performing standard membrane feeding assays (Ponnudurai et al., 1989) and standard mosquito infections by feeding on infected mice, respectively. Female gametes/zygotes and ookinetes in mosquito midguts were counted after staining either with FITC-labeled Pfs25-specific mAbs for *P. falciparum* or with FITC-labeled Pbs21-specific mAb for *P. berghei.* Oocysts were counted 7–10 days after mosquito infection.

Gamete production, fertilization, and zygote development of Pbs48/45ko parasites were determined in an in vitro fertilization assay (Janse et al., 1985a, 1985b). The fertility of male and female ko gametes was determined by cross-fertilization of different clones of *P. berghei* in the fertilization assays. Cross-fertilization between gametes of two clones (A and B) was obtained as follows: infected blood from a mouse infected with clone A and infected blood from a mouse infected with clone B were separately transferred into ookinete culture medium to induce gamete formation. 10 min after induction, equal numbers of gametes of both clones were mixed and 18 hr later, the number of ookinetes and unfertilized gametes were counted. To specifically block male gamete formation in one of the two clones, Aphidicolin at a concentration of 5×10^{-4} M was added to the cultures during the first 10 min of gamete induction (Janse et al., 1986). Further detail on the precise methods used in the phenotype analysis is available at http://www.cell.com/cgi/content/full/104/1/153/DC1.

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GenBank Accession Number

The GenBank accession number for the entire nucleotide sequence of the ORFs of *pbs47* and *pbs48/45* and their intergenic regions is AF314253.