A protective merozoite protein of *Plasmodium falciparum* shares an epitope with surface antigens of *Paramecium*

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**SUMMARY**

*A Plasmodium falciparum* cDNA expression clone, λPf9, had been identified earlier as a protective epitope, using anti-λPf9 antibodies and combinatorial phagotopes. A segment of the Pf9 gene showed homology with *Paramecium* immobilization surface antigens such as 51B, 51A and 156G. A synthetic Pf9-peptide was designed from this region, and specific antibodies were raised. Each of these anti-Pf9 antibodies and combinatorial reagents, as well as anti-Paramecium 51B antibodies, recognized the Pf9-peptide on ELISA, and the same protein band in parasite immunoblots. The *P. falciparum* protein was released from the merozoite membrane fraction on treatment with PI-PLC, indicating the presence of a GPI anchor. Anti-Pf9-peptide antibodies specifically inhibited the growth of *P. falciparum* in culture. Immunofluorescence assays showed the reactivity of anti-Pf9-peptide sera with *P. falciparum* merozoites and gametocytes, as well as on the surface of Paramecium tetraurelia. The Pf9-peptide was able to induce proliferation of splenic lymphocytes obtained from mice infected with the rodent malarial parasites *Plasmodium berghei* and *Plasmodium yoelii*. These results point towards *Plasmodium* Pf9 as a conserved novel protective protein, sharing an epitope with *Paramecium* surface antigens.

**Keywords** *Paramecium*, *Plasmodium*, protective surface antigen

**INTRODUCTION**

Malaria continues to be a major parasitic disease responsible for extensive morbidity and mortality in the tropical and subtropical areas. The complex lifecycle of the malarial parasite *Plasmodium falciparum*, the diversity of its surface antigens (1), the ability of the parasites to cytoadhere and undergo frequent antigenic variations (2), are some of the reasons why an effective malaria vaccine is yet to emerge. However, naturally acquired immunity against *P. falciparum* malaria has been well documented in residents of malaria-endemic areas (3). A large number of studies have shown the protective properties of immunoglobulins from malaria-immune adults (4–6). Efforts to identify the protective immune antibodies and their target malarial epitopes have led to the identification of proteins with repeat motifs or proteins that display variation across different strains (1). Such motifs have been postulated to play a role in immune–evasion mechanisms (7). With the aim of identifying protective malarial epitopes, a differential immunoscreen was carried out in our laboratory, which identified several cDNA clones (8). Of these, the immunoclone λP9 has been partially characterized (9). In this earlier study λP9 was shown to contain a 315 bp cDNA insert, which showed a single band on Southern blot analysis using different strains of *P. falciparum*. Combinatorial phagotopes and antibodies obtained against λP9 expression protein recognized a protein of 50 kDa in *P. falciparum* and 53 kDa in the rodent malarial parasite *P. berghei* (9). In an effort to obtain an insight regarding the possible role and the immunological relevance of Pf9 protein, a homology search was performed which revealed a weak homology of this epitope with a class of *Paramecium* surface proteins.

*Paramecium* is a free-living member of the phylum Ciliophora. The cell coat of *Paramecium* is mainly composed of a 250–300 kDa glycosylphosphatidylinositol (GPI) anchored glycoprotein called ‘surface antigen’ (SAg) that undergoes antigenic variation (10). The subtype of the SAg expressed depends on the ambient parameters such as ionic strength, pH, nutrient content, temperature, etc. (11). With a change
in any of these conditions, there is a ‘switch’ in the expressed antigen (12). Upon incubation with low concentrations of antisera against the homologous SAg the Paramecia become immobilized, while at high concentrations of antisera they die. Because of the immobilizing effect of the antibodies, these SAGs are also called immobilization antigens. In order to test whether the Plasmodium Pf9 protein is similar to these SAg proteins of Paramecium, a synthetic peptide was designed on the basis of this homology and polyclonal rabbit sera were raised against it. In this paper we show that Pf9 protein is a protective Plasmodium protein, and that it shares a common epitope with the Paramecium SAg proteins.

MATERIALS AND METHODS

Plasmodium falciparum strains and cultures

Blood stage asexual stages of the 3D7 strain of the parasite were cultured in vitro as described earlier (13). The P. berghei ANKA and P. yoelii 17XL strains were maintained by passaging asexual stages through BALB/c mice by intraperitoneal injections (14). Parasitaemia was monitored by making periodic peripheral blood smears from infected mice (13). Parasite cultures were synchronized by sorbitol treatment according to the method of (15). Paramecium tetraurelia strain 51 was obtained from J. L. van Houten (University of Vermont, Burlington, USA). About 10^5–10^6 cells/mL were coated on slides, air-dried, fixed with chilled methanol, permeabilized using 0·15% saponin and processed for IFA.

Design of Pf9 peptide and anti-Pf9 reagents

A NCBI Blast search carried out with translated Pf9 sequence showed a 30% homology with a class of Paramecium surface proteins (Figure 1). A peptide NH₂-RTCLDAAWWTDDK-MCS-COOH (Pf9-peptide) was designed from the region of maximum homology, and procured from Mimotopes (Australia). This Pf9-peptide was coupled with BSA using sorbitol treatment according to the method of (16). The sera, collected after the third and fourth boosts, were pooled, and cleared repeatedly with nitrocellulose coated with λPf9 recombinant protein, whereas combinatorial phagotopes were generated by panning combinatorial peptide phage display libraries against λPf9 recombinant protein (9).

ELISA

The ELISA assay was carried out using standard protocols. The peptides were coated on Maxisorp plates (Nunc, Roskilde, Denmark) at 1 µg/well concentration. Phagotopes were biotinylated as described earlier (9).

Immunoblots

Intracellular parasites from the asexual stages were liberated from infected erythrocytes by saponin lysis (0·15%), and the pellet was washed with PBS (9). The total crude parasite extract was used for Western blot analysis. For merozoite preparation, a synchronized culture was harvested at the mature schizont stage, and cultured at 37°C for 4 h. The red cells were then pelleted at 800 g for 10 min, and the supernatant was centrifuged at 4000 g for 30 min to get the merozoite pellet (17). This pellet was boiled with SDS-lysis buffer with or without β-mercaptoethanol to obtain reduced or unreduced protein preparations. The samples were separated on a 10% SDS-PAGE, and blotted onto Hybond-C membrane (Amersham), and immunoscreened with appropriate antibodies. Theuffy coat, obtained from human peripheral blood cells, was used as the source of crude human protein extract (18).

PI-PLC treatment

Merozoites were prepared as described above and treated with 2 U/mL PI-PLC (Bacillus cereus, Glykomart) as described for Paramecium (19). Briefly, treated and untreated aliquots of merozoites (10^5 merozoites per treatment) were incubated in 0·5 mL at 4°C for 4 h on a shaker. The samples were centrifuged at 4000 g for 30 min at 4°C. The pellet and

Figure 1 Box shading showing homology of Pf9 cDNA ORF with 51B (U07603) and 51A (M65163) surface antigens of Paramecium tetraurelia, and 156G (X03882) surface antigen from Paramecium primaurelia. The underlined sequence defines the synthetic Pf9-peptide.
supernatant fractions were collected separately and tested on immunoblots with anti-Pf9-peptide antibodies. The band intensity for each treatment was calculated using Metamorph Version 4.6 (Universal Imaging Corporation, PA, USA).

**Invasion blocking assay**

This assay was performed as described in (13). Briefly, *P. falciparum* was grown in vitro and synchronized by repeated sorbitol treatment (15). The culture was harvested at early ring stage and subjected to the assay at a parasitaemia of 2–3% with 5% haematocrit. IgG was purified from pooled rabbit sera as described earlier (13) and added to the culture at a concentration of 0·2 mg/mL Pf9-peptide was added along with the IgG.

**Immunofluorescence assay**

IFA was performed with *P. falciparum* (3D7) and human leucocytes as described earlier (9). The slides were treated with anti-Pf9-peptide sera, anti-MSP-1 serum (gift of Tony Holder) and pre-immune serum at a dilution of 1:50. *Paramecium tetraurelia* cells were fixed on IFA slides using chilled methanol and then treated in the same way. Alexa Fluor 488-conjugated anti-rabbit immunoglobulins (Molecular Probes) were used as secondary antibodies. Confocal microscopy was performed using a Bio-Rad Radiance 2000 imaging system mounted on a Nikon inverted microscope. An argon laser was used as the excitation source at 488 nm. The samples were viewed using a 100× oil immersion objective lens.

**Lymphocyte proliferation assay**

The assay was carried out in a 96-well plate (Nunc, Roskilde, Denmark) as described earlier (9). Briefly, a cell suspension was made from the spleens of two uninfected mice and two mice infected with *P. yoelii*. The suspension was cleared of cell debris and red cells, followed by incubation for 48 h at 37°C in a 5% CO₂ incubator (Forma, USA). Different antigens (Pf9 peptide, BSA) or a mitogen (Concanavalin A) were then added to the wells in triplicate at different concentrations. The cells were allowed to incubate further for 2–4 days and then 2 μCi of tritiated thymidine (BRIT, India) was added. The cells were harvested after 2 days and the amount of thymidine incorporation was measured using a liquid scintillation counter (LKB, Finland).

**Results**

**Reactivity of anti-Pf9 reagents with Pf9 peptide**

A homology search with the translated Pf9 gene sequence using low stringency search conditions (20) showed a weak homology with the immobilization antigens of the *Paramecium* (Figure 1). These SAg of *Paramecium* are cysteine-rich proteins, and the *Plasmodium* ORF also has five cysteine groups in the small stretch of 49 amino acids, at fairly conserved distances. The identities and similarities between Pf9 and *P. falciparum* 51B antigen over this segment of 49 amino acids were 13/49 (27%) and 15/49 (31%). Since the homology was much weaker than normally allowed for orthologous proteins (~30% identity and 40% similarity), it was imperative to test the validity of this homology. Therefore the Pf9-peptide was synthesized (Figure 1), and specific antiseras were raised against this peptide.

We have earlier reported the use of different reagents, anti-λPf9 sera and λPf9-specific phagotopes, for the characterization of the λPf9 expression clone (9). To test whether these different anti-Pf9 reagents recognize the synthetic Pf9-peptide, ELISA was carried out using Pf9-peptide as the antigen. λPf9-specific phagotopes, the anti-λPf9 sera as well as the pooled anti-Pf9-peptide serum, showed reactivity with the Pf9-peptide over and above the wild-type phage (M13) or the pre-immune sera values (Figure 2a). The anti-Pf9-peptide serum and the polyclonal serum against the *Paramecium* 51B protein (gift from Dr J. Forney, Purdue University) reacted with Pf9 peptide, but not with the irrelevant 15-mer peptide from the insulin β-chain (Figure 2b). In the presence of λPf9-specific phagotopes, anti-Pf9-peptide serum activity was reduced, showing that λPf9-specific phagotopes compete with the anti-Pf9-peptide serum. The anti-Pf9-peptide sera also recognized the λPf9 expression protein on an immuno-dot blot assay (data not shown). These results show that all three classes of reagent against the *Plasmodium* Pf9 epitope, as well as the anti-Paramecium 51B protein antibodies, recognized the synthetic Pf9-peptide.

**Immunoblot analysis of parasite protein extracts and PI-PLC assay**

Anti-λPf9 sera and λPf9-specific phagotopes have been shown earlier to recognize a band of 50 kDa in *P. falciparum* immunoblots and a band of 53 kDa in the rodent malarial parasite *P. berghei* immunoblots (9). The anti-Pf9-peptide antibodies also recognized the same bands of 50 and 53 kDa in *P. falciparum* and *P. berghei* immunoblots, respectively (Figure 3a). A 53 kDa protein band was also detected in the rodent malarial parasite *P. yoelii* blots (Figure 3a). In the presence of the Pf9 peptide, the reactivity of this 53 kDa
Figure 2 ELISA reactivity with plates coated with Pf9-peptide (1 µg/well). (a) Reactivity of different Pf9 specific reagents with Pf9 peptide. Phagotopes and M13 phages were biotinylated and reactivity monitored using streptavidin HRP. Anti-Pf9-peptide, anti-λPf9 and the pre-immune serum PI were checked for binding using anti-rabbit secondary antibodies. The assay was carried out with different concentrations of phagotopes, $10^5$ and $10^6$, anti-Pf9-peptide sera and pre-immune sera at 1 : 3000 (open bar) and 1 : 1000 (hatched bar), and anti-λPf9 sera 1 : 1000 (open bar) and 1 : 500 (hatched bar), respectively. (b) Reactivity of anti-Paramecium-51B (51B); anti-Pf9 peptide (Pf9); and preimmune serum (PI Pf9) antibodies with Pf9 peptide. The sera were tested at two different concentrations for binding to Pf9 peptide (Pf9 pep) and an irrelevant 15-mer peptide from insulin β-chain (irr pep). Both peptides were used at 1 µg/well.

Figure 3 Immunoblot analysis using total protein extracts from P. falciparum (PF), P. berghei (PB), P. yoelii (PY) and human leucocytes (HS). (a) 10 µg each of parasite protein extract from a mixture of rings, trophozoites and schizonts, and human leucocytes was resolved on a 10% SDS-PAGE under reduced conditions. (b) Immunoblot analysis of P. falciparum merozoite protein extract. The samples were resolved under both reduced (R) and unreduced (U) conditions. The immunoblots were treated with different antibodies as indicated on the top of each strip. PI: pre-immune serum; α-Pf9: anti-Pf9-peptide antibody; α-51B: anti-Paramecium-51B antibody; P9/α-Pf9: anti-Pf9-peptide antibody in the presence of 50 µg/mL of Pf9 peptide. Anti-Pf9-peptide and pre-immune sera were used at 1 : 200 dilution and anti-51B antibodies were used at 1 : 100 dilution. The numbers on the left indicate the molecular weight markers. The arrowheads show the 50 kDa P. falciparum and the 53 kDa P. berghei and P. yoelii proteins.
band decreased considerably, demonstrating a competition between the peptide and the 53 kDa *P. yoelii* protein. Antiserum against the 51B protein of *Paramecium tetraurelia* also recognized the same size *Plasmodium* proteins in the respective immunoblots (Figure 3a). These results were obtained with parasite protein prepared from asynchronuous cultures containing a mixture of ring, trophozoite and schizont stages. Immunoprecipitation assays, using anti-Pf9-peptide sera and *P. falciparum* extracts from a mature schizont preparation, precipitated the same 50 kDa protein (data not shown). To test for the presence of any cross-reactive protein in human samples, human leucocyte protein preparation was tested with the sera and no reactivity was detected (Figure 3a).

The anti-Pf9-peptide serum detected the 50 kDa protein in *P. falciparum* merozoite preparation as well (Figure 3b). The *Paramecium* SAg proteins possess a large number of cysteine residues, and the recognition of the *Paramecium* protein is found to be better when the protein preparation is reduced (21). Therefore, the *P. falciparum* merozoite preparation was immunoscreened under both reduced and unreduced conditions (Figure 3b). No significant difference was observed in the reactivity of the anti-Pf9-peptide sera. There was, however, a considerable reduction in the recognition of the protein by anti-51B protein sera under unreduced conditions (Figure 3b). This indicates that there may be additional cysteine-rich domain(s) in the 50 kDa *Plasmodium* protein, which are preferentially detected by the anti-51B antibodies under reduced conditions.

Since the *Paramecium* SAg are GPI-anchored proteins, and since GPI anchors can be cleaved from the cell surface by PI-PLC treatment, it was decided to test for such a cleavage for Pf9 protein. Upon treatment of *P. falciparum* merozoites with PI-PLC, the amount of 50 kDa Pf9 protein in the supernatant was found to increase significantly (Figure 4). Similar treatment did not release another merozoite-associated PfP0 protein, indicating the absence of non-specific protease activity in the PI-PLC enzyme preparation. A quantitative image analysis of the blots, using independent experiments, showed this increase to be about 2.5 to 3-fold over the untreated sample (Figure 4). After PI-PLC treatment, 80% of the protein was present in the supernatant, whereas before the PI-PLC treatment this proportion was found to be around 30% (Figure 4). This result indicates that the 50 kDa protein may be GPI-anchored.

**Localization of Pf9 protein**

Immunofluorescence assay performed with mature schizonts and gametocytes shows the presence of the Pf9 epitope on the merozoite and gametocyte periphery (Figure 5I). On the mature schizont stage, the pattern of expression was similar to merozoite surface protein 1, which is one of the major surface proteins of the merozoite stage. Human peripheral blood cells (red blood cells as well as leucocytes) did not show any cross-reactivity with anti-Pf9-peptide sera (data not shown). To test whether anti-Pf9-peptide antibody would react to the *Paramecium* cell surface, IFA was carried out on fixed *Paramecium tetraurelia* cells (Figure 5II). The results showed that the surface reactivity of the Pf9 epitope was dominant. However, unlike the 51B reactivity, the Pf9 reactivity was not limited to the surface. This feature is also observed in *Plasmodium* schizonts and gametocytes, as punctate IFA reactivity was detected inside the cells (Figure 5I).

**Invasion blocking assay**

Anti αPf9 reagents have been shown to inhibit the growth of *P. falciparum* (9). To test for invasion blocking, a *P. falciparum* culture was synchronized to the ring stages, and treated with purified anti-Pf9-peptide IgG (Figure 6). There was no effect of the IgG during the intra-erythrocytic development of the parasite from rings to trophozoite and schizont stages, up to 42 h. However, at the 48 h time point, there was a distinct reduction in parasitaemia as compared to the controls. This is the phase when released merozoites invade fresh red blood cells, giving rise to ring stages. The reduction at this stage indicated a block specifically at the step of invasion of red cells by merozoites. The inhibition could be competed out specifically using 0.2 mg/mL Pf9 peptide.
Lymphocyte proliferation assay

In order to study the ability of the Pf9-peptide to elicit T cell proliferation, mice were infected with the rodent malarial parasite *P. yoelii* and the proliferation of the lymphocytes from infected and uninfected mice was measured in response to different antigens. About three- to 10-fold stimulation of cells were observed with Pf9-peptide over and above any non-specific stimulation, monitored by using BSA as an irrelevant antigen source (Figure 7). This response is not seen with uninfected mouse lymphocytes. However, the uninfected mouse lymphocytes proliferated in response to the mitogen Concanavalin A, demonstrating the viability of these cells. These studies establish that the *P. yoelii* parasites elicit a cellular immune response against the Pf9-peptide epitope in mice during the course of infection. This assay also affirms that this immune epitope is conserved between the human malarial parasite *P. falciparum* and the rodent malarial parasite *P. yoelii*.

DISCUSSION

The preliminary characterization of the Pf9 gene and the corresponding parasite protein has been reported earlier (9). Through Southern blot and PCR data it was shown that a single *P. falciparum* gene codes for Pf9. However, several attempts to recover the flanking regions using inverse PCR were met with failure. So far the *Plasmodium* database has not shown up the presence of this gene segment. Perhaps the flanking template DNA sequences form complex folded...
secondary structures, which are inaccessible to the primers or to the polymerase enzyme. There are still gaps in the *P. falciparum* genome sequence (22) and possibly Pf9 gene falls in such a gap.

The data presented here support the homology between the Pf9 epitope and *Paramecium* surface antigen. ELISA, immunobLOTS and competition assays using three different classes of anti-Pf9 reagents, as well as anti-51B *Paramecium* protein, show that despite the varied origin of these reagents, each reagent recognized the Pf9-peptide epitope, and identified a 50 kDa protein from the human malarial parasite *P. falciparum*, and a 53 kDa protein from each of the rodent malarial parasites *P. berghei* and *P. yoelii*. Immunofluorescence assays established the presence of this epitope on the merozoite and gametocyte surface of *P. falciparum*. In invasion blocking assays, the block occurred specifically at the merozoite stage, prior to the invasion of red blood cells. The lymphocyte proliferation data demonstrated that lymphocytes specific for the Pf9-peptide are activated during the course of a rodent malarial parasite infection in mice.

Surface antigen genes in *Paramecium tetraurelia* constitute a multigene family of distinct, unlinked loci (11). They constitute 25% of total membrane protein and vary in molecular weight from 250 to 300 kDa. The proteins are recognized according to the strain from which they have been isolated, such as 51A and 51B from strain 51, and 156G from strain 156. The expression of a specific type appears to be governed by environmental factors such as temperature, pH, ionic strength of the media, proteolytic enzymes, UV rays, etc. (24). Although considerable information is available about the expression patterns and regulation of these subtypes (25), the function(s) of these surface proteins still remain to be worked out. There have been postulates about the role of these proteins in cell signalling, so that in different environmental conditions, different expressed antigen may function optimally (26). The SAgs may also be playing a defensive role against various abiotic and biotic stresses (27).

From the Western blot data obtained with *P. falciparum* merozoites treated with PI-PLC, it appears that the Pf9 protein has a PI-PLC sensitive GPI-anchor. It has been reported that malarial GPs are unaffected by treatment with phosphatidylinositol-specific phospholipase C (28). However, these are bulk studies and cannot rule out the possibility of a few PI-PLC sensitive GPI-anchored proteins. PI-PLC mediated release of a 76 kDa *Plasmodium falciparum* membrane protein from intact merozoites has been reported earlier (29). Recently, a set of lower molecular weight (40–60 kDa) GPI-anchored surface proteins, referred to as cross-reacting glycoproteins (CRG), has been reported in *Paramecium primaurelia* (30). The gene and protein sequences for these lower molecular weight proteins are not known as yet. No specific functions have been attributed to these CRG. However, unlike the variable SAg of *Paramecium*, these CRG were found to be present in all strains of *Paramecium* tested. Using mutant strains of *Paramecium*, it has been shown that these lower molecular weight proteins are not proteolytic products of the immobilization antigens (30). GPI-anchored proteins are known to play a role in signal transduction after cleavage with PI-PLC. Since these are conserved GPI-anchored surface proteins in *Paramecium*, and are of a size comparable to Pf9, it is tempting to speculate that Pf9 protein and these *Paramecium* lower molecular weight proteins play similar roles in these unicellular protozoaNS. It is, however, also possible that the 50 kDa Pf9 protein may not be GPI-anchored, but is linked with a GPI-anchored protein on the merozoite surface.

The 50 kDa *P. falciparum* Pf9 protein is a merozoite protein, capable of eliciting both B- and T-cell-mediated immune responses. Eight merozoite surface proteins (MSP) of *Plasmodium falciparum* have been reported so far (31). Some of
these MSP proteins are polymorphic amongst different strains of *P. falciparum* and are typically used for strain typing (32). Of these MSPs, five are reported to be GPI-anchored proteins (31). Some of these GPI-anchored MSP proteins are conserved across different strains of *P. falciparum* (33). Antibodies against the rodent homologue of MSP 4 and 5, termed MSP 4/5, are cross-reactive amongst different rodent malarial parasite species (34). Data presented in this paper show that Pf9 is one such epitope conserved amongst human and rodent parasite species. Sequence comparison between known MSP genes and the Pf9 sequence shows that Pf9 is different from these MSP proteins.

In the differential immunoscreen in which Pf9 was identified (8), other cDNA clones were also found to code for proteins conserved across species, such as the ribosomal phosphoprotein P0 (35). The P0 protein was found to be protective (14), but it suffers from the disadvantage of cross-reacting with corresponding human P0 protein (18). Pf9 epitope does not appear to show any cross-reactivity with human proteins, and is therefore a potential candidate for a malarial vaccine. An elucidation of the complete protein sequence of Pf9, and better understanding of the function of such conserved GPI-anchored surface proteins, would help in the global effort to control malaria.

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REFERENCES


