

MINIREVIEW

***Plasmodium* P25 and P28 surface proteins: potential transmission-blocking vaccines**

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Malaria arises from the infection of red blood cells by protozoan parasites of the genus *Plasmodium* that are transmitted by anopheline mosquitoes. More than 400 species of *Anopheles* mosquitoes are known, of which about 40 species are characterized as important disease vectors for human malaria transmission (28). It is estimated that 300-500 million cases of malaria and over one million deaths from the disease occur each year (48).

The *Plasmodium* parasite must complete its development in the mosquito before it can be transmitted to the vertebrate host and cause malaria. Each stage of parasite development in the mosquito offers potential targets to interfere with malaria

1 transmission. Development of the malaria parasite in the mosquito begins when the
2 gametocyte forms of the parasite are picked up by the mosquito in the blood meal from
3 an infected human and quickly develop into extracellular gametes in the mosquito
4 midgut. After fertilization, round shaped zygotes form and transform into banana-shaped
5 ookinetes. The ookinetes are motile and must exit the gut by crossing the peritrophic
6 membrane and midgut epithelium. On the basal side of the epithelium, surviving
7 ookinetes lodge against the basal lamina, and transform into spherical oocysts. In the
8 oocyst, the parasite develops into several thousand sporozoites, which then exit the
9 oocyst and are carried by the hemolymph to the mosquito's salivary glands to infect
10 another host (65).

11 There is ongoing research to develop anti-parasite liver stage and asexual blood
12 stage vaccines against each stage of the complicated life cycle of *Plasmodium* (17, 23).
13 Liver stage vaccines are intended to reduce infection rates and asexual blood stage
14 vaccines will reduce disease severity and the risk of death during infection.
15 Transmission-blocking vaccines would prevent the spread of disease by targeting
16 antigens expressed in the mosquito stage on the surface of the gametocyte, gamete,
17 zygote, and ookinete forms of the parasite (6, 60). These vaccines induce antibodies in
18 the human host that inhibit parasite development in the mosquito midgut and thereby
19 block parasite transmission to another person.

20 This article will review the biology and structural knowledge of *Plasmodium* P25
21 and P28 proteins and their contributions in transmission blocking vaccine development.

TARGET ANTIGENS AND TRANSMISSION BLOCKING IMMUNITY

Target antigens.

The target antigens for transmission blocking vaccines are divided in two groups: pre-fertilization and post-fertilization parasite surface proteins. Pre-fertilization antigens are proteins expressed on the surface of male and female gametocytes and gametes, for example, the P48/45 and P230 proteins (40). These antigens have a unique repeated six-cysteine disulfide bonded structure (53). Monoclonal antibodies against either of these proteins can block the infectivity of the gametes to the mosquito (7, 39, 62) and the blocking activities are enhanced by complement (38).

Post-fertilization antigens are proteins expressed on the surface of zygotes and the maturing ookinete form of the parasite (31, 35, 62). The P25 and P28 proteins have been cloned from several *Plasmodium* species (14, 15, 26, 27, 35, 52, 57-59). Low-level expression of P25 is detectable in early gametocytes and the expression level dramatically increases after fertilization (62). Anti-P25 antibodies bound specifically to the surface of parasites ranging from zygotes to ookinetes. P28 is expressed slightly later in development, as anti-P28 antibodies stained mainly the retort and mature forms of ookinetes (21). P25 and P28 are distributed evenly and abundantly over the entire ookinete surface as seen by immunofluorescence antibody staining (66) and immunogold electron microscopy (15, 47). *Plasmodium* P25 and P28 proteins are the targets of effective transmission-blocking antibodies that inhibit oocyst development in mosquito gut. When a mixture of infected blood and antisera against *Plasmodium* P25 and P28 proteins is fed to mosquitoes through a laboratory membrane feeder, a significant reduction in oocyst numbers is observed (9, 21).

1 In addition to P25 and P28 proteins, other ookinete proteins have been identified
2 that are important in ookinete-to-oocyst development. These proteins are (i) parasite-
3 produced chitinase, a potential target of malaria transmission blocking intervention (42).
4 Chitinase-disrupted *P. falciparum* parasites are significantly impaired in their ability to
5 form oocysts in the mosquito gut (56). (ii) CTRP, circumsporozoite-and thrombo-
6 spondin-related adhesive protein, present in the ookinete micronemes and essential for
7 ookinete invasion and oocyst formation in the mosquito midgut epithelium (12, 54, 69),
8 (iii) Pbsub2, a subtilisin-like protease (19), (iv) WARP, von Willebrand factor A domain-
9 related protein, a secreted protein with adhesive properties of unknown function (70), (v)
10 MAOP, membrane-attack ookinete protein, which contains a perforin-related domain
11 (22), and (vi) SOAP, secreted ookinete adhesive protein, which contains two unique
12 cysteine rich domains and interacts with laminin (13). Ookinetes that were deficient in
13 SOAP exhibited significantly reduced midgut invasion and oocyst formation (13).

14 **Transmission blocking immunity.**

15 Transmission blocking immunity can be mediated by antibodies against parasite surface
16 proteins, which act in the midgut of a blood-fed mosquito. The P25 and P28 proteins are
17 only expressed in the mosquito. These proteins normally do not encounter the human
18 immune system, but antibodies raised against recombinant P25 and P28 proteins, when
19 taken up by mosquitoes, stop parasite development in the mosquito gut.

20 Several transmission-blocking vaccine formulations are being developed using *P.*
21 *vivax* and *P. falciparum* P25 and P28 proteins produced in yeast. Antisera from mice
22 immunized with recombinant Pvs25 (P25 from *P. vivax* parasites) completely prevented
23 the appearance of oocysts in mosquitoes that had ingested the antisera with *P. vivax*

1 parasites (21). In a Phase I vaccine trial of Pvs25 bound to aluminum hydroxide, the
2 levels of antibodies that were generated correlated with transmission blocking activity
3 (33). Antibodies obtained after immunization of mice and monkeys with yeast-produced
4 Pfs25 (P25 from *P. falciparum* parasites) (73) have shown significant transmission
5 blocking activity in experiments, in which a mixture of antisera, blood, and parasite
6 cultures were fed to the mosquitoes through a membrane feeding apparatus (25). In
7 humans, priming with a Pfs25 gene-containing vaccinia virus and boosting with Pfs25
8 protein yielded antisera with significant transmission-blocking activity (24). Covalent
9 conjugation of P25 proteins by chemical cross-linking to carrier proteins is a promising
10 strategy as it yields strong and sustained antibody responses (30, 36, 67).

11 **INTERACTIONS OF P25 AND P28 WITH MOSQUITO MIDGUT PROTEINS**

12 The interaction of ookinetes with the basal lamina is important for ookinete invasion and
13 oocyst development in the mosquito. *Plasmodium* P25 and P28 proteins play an
14 important role in parasite recognition of and attachment to the mosquito midgut (44, 45,
15 55). *P. berghei* P25 and P28 proteins were shown, by yeast two-hybrid experiments, to
16 interact with laminin, a major constituent of the basal lamina surrounding the midgut of
17 *Anopheles gambiae* (63). *P. gallinaceum* P25 and P28 proteins interact with midgut
18 basement membrane in order to attach the parasite to its surface (1). The P25 of *P.*
19 *berghei* binds to laminin and collagen IV and the binding is involved in the
20 transformation of ookinetes into oocysts (3).

21 A study that combined knowledge of the sequenced genomes of *Drosophila*
22 *melanogaster* and *A. gambiae* identified annexin proteins, which bind to *P. berghei*
23 ookinetes during invasion of the mosquito midgut and play important roles in mosquito
24

1 infection (29). When a blood meal containing a mixture of *P. berghei* parasite and anti-
2 annexin serum were fed to mosquitoes in membrane feeding experiments, the number of
3 observed oocysts was considerably reduced, compared to control. Confocal analysis of
4 dissected midguts with anti-anopheles annexin mouse serum and the 13.1 monoclonal
5 antibody recognizing *P. berghei* P28 revealed that the staining of P28 and annexin
6 overlapped and so the two proteins co-localized (29).

7 8 **GENE DISRUPTION STUDIES** 9

10 Gene disruptions of P25 and P28 reveal that the two proteins have partially redundant
11 functions in parasites and are involved in ookinete survival in midgut, penetration of the
12 midgut epithelium, and the transformation of ookinetes to oocysts (55). When blood
13 infected with *P. berghei* having either the P25 or the P28 gene disrupted, was fed to
14 mosquitoes through a membrane feeder, oocyst formation was slightly affected as
15 compared to oocyst formation in mosquitoes infected with wild-type *P. berghei*.
16 However, when both genes were knocked out at the same time, almost no oocysts were
17 formed (55). The ookinetes in the double-knockout parasite were swollen in appearance
18 and did not cluster together in the gut, as they do in wild-type parasites (55). A recent
19 study on midgut epithelium invasion by double-knockout *P. berghei* ookinetes implies
20 that the loss of P25/P28 proteins greatly reduced, but did not entirely prevent, the entry of
21 ookinetes into midgut epithelial cells (5).

STRUCTURAL STUDIES

Primary structure.

Plasmodium P25 and P28 proteins are evolutionarily conserved and are comprised of a predicted signal sequence at their N-termini, followed by four epidermal growth factor (EGF)-like domains, and a C-terminal glycosyl phosphatidyl inositol moiety that anchors the proteins to the parasite surface (23, 26). Sequence analysis shows that P25 proteins contain 22 cysteine residues held together with 11 disulfide bonds and P28 proteins contain 20 cysteine residues with 10 disulfide bonds. EGF-like domains are predominantly found in extracellular proteins of eukaryotes, where they participate in adhesion and signaling (2). A typical EGF-like domain contains 40-50 residues including six cysteines that form disulfide bonds in the pattern 1-3, 2-4, and 5-6. EGF-domains contain a variable number of residues between the cysteines except for the single residue between cysteines 4 and 5.

X-ray crystal structure of Pvs25.

The structure determination of Pvs25 used the same yeast-produced recombinant protein as is used for vaccine trials (33, 41). It was the first structure of a *Plasmodium* surface protein from the mosquito stage and revealed the unprecedented arrangement of the four EGF-like domains of Pvs25 to form a compact triangular prism. In the Pvs25 crystal, triangular prisms are arranged as layers of sheets. Pvs25 residues that form inter-domain contacts within the molecule and inter-molecular contacts involved in sheet formation in the crystal are highly conserved in P25 and P28 proteins from all *Plasmodium* species (41).

1 Examination of the *P. falciparum* P25 sequence revealed that the Pfs25 protein
2 likely assumes the same triangular structure as Pvs25. There is complete conservation of
3 the residues forming the contacts among EGF-like domains 1, 3, and 4 that bring the four
4 domains into their shape. The overall sequence identity between Pfs25 and Pvs25 is
5 46%, yet Pfs25 has been predicted to be similar to Pvs25 due to the disulfide bonding
6 similarities of the EGF-like domains. Pvs25 and Pvs28 are related, exhibiting 41% amino
7 acid sequence identity over 157 residues of Pvs28. The residues that interact between
8 domain 1 and domains 3 and 4 in Pvs25 are conserved throughout the sequences of the
9 P28 family. Therefore, the structure of Pvs25 will likely be a valid model for the
10 structures of all P28 family members (41).

11 **Sequence polymorphisms.**

12 There are relatively few sequence polymorphisms in P25 and P28 that are isolated from
13 *P. falciparum* and *P. vivax* populations in the field, presumably because the P25 and P28
14 proteins are not expressed in the vertebrate host and thus are not exposed to selection
15 pressure from the vertebrate immune system (8). In Pfs25, two conserved amino acid
16 substitutions and two silent changes were found, while in Pfs28 protein a Lys to Arg
17 change at position 72 had been found and recently, a new non-synonymous substitution
18 (Asp to Ala at position 104) was found through the genome-wide single nucleotide
19 polymorphism (SNP) analysis (18, 27, 43) (www.plasmodb.org, PF10_0302). A study
20 conducted on *P. vivax* Sal I strain, using one isolate from India and two isolates from
21 Bangladesh, indicates that the Pvs25 protein contains only three point mutations that
22 would result in amino acid substitutions while Pvs28 gene had 22 point mutations, but all
23 were conserved substitutions (59). The most striking variation was detected in an Indian

1 isolate of Pvs28 that consists of four C-terminal tandem repeats of GlySerGlyGlyGlu/Asp
2 instead of the usual six repeats found elsewhere (59).

3 4 **BINDING OF TRANSMISSION BLOCKING ANTIBODIES** 5

6 In the mosquito gut, parasites exit red blood cells and thus become vulnerable to anti-
7 parasite antibodies in the blood meal. Studies in mice, rabbits, and rhesus monkeys
8 demonstrated that yeast-expressed Pvs25 formulated on aluminum hydroxide gel induces
9 antibodies that block development of *P. vivax* in mosquitoes as demonstrated in the ex-
10 vivo membrane-feeding assay (6, 21). When antisera from mice immunized with
11 individual domains of *P. falciparum* P25 were mixed with infected blood and fed to
12 mosquitoes in the laboratory, EGF-like domain 2 antisera had the highest transmission-
13 blocking activity (50). In *P. berghei*, the transmission-blocking mAb 13.1 against P28
14 was mapped using deletions and overlapping peptides to the sequence
15 GlyLeuGluLysAlaPheValCys on the B loop of domain 2 (49).

16 EGF-like domain 3 is the target of other transmission-blocking mAbs. The mAbs
17 4B7 and 1D2 recognized domain 3 in enzyme-linked immunosorbent assays using the
18 individually expressed domains of *P. falciparum* P25 (50). The 4B7 binding site was
19 mapped to the sequence LeuAspThrSerAsnProValLys at the apex of the B loop of EGF-
20 like domain 3, using overlapping synthetic peptides from the *P. falciparum* P25 sequence
21 (51). By similar methods, the independently generated mAb 32F81 against *P. falciparum*
22 P25 was mapped to the same site on the B loop of domain 3 (61).

23 To investigate the place and mode of binding of a transmission-blocking antibody
24 to Pvs25, the structure of a Fab fragment of a transmission-blocking antibody bound to
25 Pvs25 was determined (41). Monoclonal antibodies were generated in mice using yeast-

1 produced Pvs25 as immunogen and were shown to bind to parasites in immuno-
2 fluorescence experiments (72). In the structure, the Fab fragment of 2A8 binds to the B
3 loop of domain 2 of Pvs25 (**Fig. 1**). Monoclonal antibodies 1H10 and 1A5 also bind near
4 or at the B loop of domain 2 as they were unable to bind Pvs25 that had been prebound
5 with saturating amounts of Fab 2A8 (41).

6 7 **POSSIBLE FUNCTIONS IN MOSQUITO MIDGUT** 8

9 **Protection.**

10 *Plasmodium* P25 and P28 proteins may play an important role in protecting the parasite
11 from the harsh proteolytic environment of the midgut and mosquito immune system.

12 When a mosquito takes a blood meal from an infected person, the P25 and P28 proteins
13 are expressed early and coat the parasite surface. Interactions between P25/P28 coated
14 cell surfaces (45) may mediate parasite clustering, as these proteins are extremely
15 abundant on ookinete surface (47). P25/P28 double knockout and antibody treated
16 ookinetes did not cluster together (45, 46, 55) in the blood meal, as do wild-type and
17 untreated ones (68). Lack of clustering arising from lessened interactions between
18 adjacent parasites may expose even the inner ookinetes of the cluster to the damaging
19 proteolytic conditions of the midgut (16).

20 The Pvs25 crystal structure revealed a triangular prism shaped structure that could
21 tile the parasite surface (41). In the crystal, Pvs25 is packed as tightly arranged sheets that
22 could also occur on parasite surface to form protective coat (**Fig. 2**). P25 and P28 can
23 substitute for each other in single knockout parasites (55). Perhaps either one alone can
24 form an effective surface sheet, but a more protective one might be formed when both

1 proteins are present. Yeast two-hybrid experiments (45) showed that Pvs25 has a
2 tendency to form dimers, a requirement for forming a sheet.

3 In five independent structural views of Pvs25, the position of the C-terminal half
4 of domain 4 pivots in the plane of the triangle so that the angle it makes with the rest of
5 domain 4 varies (A. K. Saxena and D. N. Garboczi, unpublished data). This variation
6 would be useful for adjusting a molecule's fit in a sheet of other cell surface molecules.

7 **Involvement with ookinete entry into midgut epithelial cells.**

8 The migration of double knockout P25/P28 parasites into and across the midgut
9 epithelium is significantly reduced, but not abolished, when compared to wild-type
10 parasites (55). A study using wild-type and double knockout *P. berghei* indicates that
11 double knockout parasites may migrate through the midgut epithelium via an intercellular
12 route rather than the intracellular route used by wild-type parasites (5). P25 and P28 may
13 play an important role in mediating ookinete entry in midgut epithelial cell (5). P28
14 protein is shed from the ookinete and is found at the site of ookinete penetration of the
15 midgut epithelial cell and tracks through the invaded cell's cytoplasm are seen (10, 20).
16 The presence of P28 protein on and in cells suggests a function in an aspect of the
17 traversal of the epithelium, though the conclusion that P25 and P28 did not play critical
18 roles in recognition or penetration of the midgut epithelium came from the double
19 knockout studies (45, 55). During invasion of the epithelium, wild-type *P. berghei*
20 ookinetes cause significant damage to cells (10, 20, 34, 64, 71), which show the loss of
21 microvilli, extension of the damaged cell into the midgut lumen, and increased expression
22 of nitric oxide synthase (20, 32) and particular serpin molecules (10, 11). Midgut cells
23 that were invaded by double knockout P25/P28 *P. berghei* do not show the abnormal

1 characteristics observed during and after invasion by wild-type *P. berghei* ookinetes (5,
2 10).

3 VACCINE DEVELOPMENT

4
5 Transmission blocking vaccines against the two major species of human malaria parasite,
6 *P. falciparum* and *P. vivax*, are under development (6, 60). Both P25 and P28 are
7 encoded by single copy genes that are highly conserved among parasite isolates. This
8 feature simplified the vaccine design, as a vaccine based on a single target gene sequence
9 will be effective for all parasite isolates in various geographic locations. On the other
10 hand, lack of expression in the human host indicated that this vaccine would not be
11 boosted by natural malaria infection. As result, the vaccine has to be highly potent to
12 induce adequate antibody levels that are sustained for at least one transmission season.
13 Vaccination of mice, rabbits, and monkeys with yeast-produced Pfs25 with aluminum
14 hydroxide as adjuvant induce transmission blocking antibodies (4, 25, 37). Yeast-
15 produced Pvs25 formulated with aluminum hydroxide as adjuvant was evaluated in a
16 Phase-I clinical trial (33). However, antibody titers produced after Pvs25 immunization
17 are low and more potent adjuvant formulations are required for inducing high antibody
18 titers. The yeast-produced Pfs25 formulated with Montanide ISA51 is in a Phase-I
19 clinical trial. Despite the evidence of Pfs25 being an effective vaccine target, the protein
20 requires potent adjuvant formulations to increase the immunogenicity to sustain high
21 antibody titer. A covalent chemical conjugate of yeast-produced Pfs25 linked to OMPC
22 (outer-membrane protein complex of *Neisseria meningitidis* serogroup B) and formulated
23 with aluminum hydroxyphosphate was more effective in generating anti-Pfs25 ELISA
24 reactivity compared to Pfs25 alone in Montanide ISA720 at the same dose (67). The

1 conjugate vaccine Pfs25-OMPC has shown sustained high immunogenicity in rhesus
2 monkeys (67). Conjugating Pfs25 to a non-toxic recombinant *Pseudomonas aeruginosa*
3 ExoProtein A (36) or to Pfs25 itself to form multimeric molecules (30) also significantly
4 increased the immunogenicity of Pfs25.

5 6 **CONCLUDING REMARKS** 7

8 Vaccines targeting the *Plasmodium* P25 and P28 proteins are promising strategies for
9 malaria control as they induce antibodies in humans that inhibit the parasite in mosquito
10 midgut. The gene knockouts show that P25 and P28 share multiple functions during
11 ookinete to oocyst development. The structure of Pvs25 from *P. vivax* is the first of a
12 mosquito stage surface protein and has a novel arrangement of EGF domains. The Pvs25
13 forms triangular prism structures and residues forming the triangles are highly conserved
14 in all other *Plasmodia* and thus, Pvs25 is a good template for predicting the structures of
15 other P25 and P28 proteins. P25 and P28 interactions with transmission-blocking
16 antibodies indicate that antibodies bind to the B loops of the second and third EGF-like
17 domains of P25 and P28 proteins. The complex of Pvs25 with a Fab fragment showed
18 how one transmission-blocking antibody binds P25; generation of such antibodies by
19 inexpensive and simple transmission-blocking vaccines should play an important role in
20 the control of malaria transmission.

21 *Plasmodium* surface proteins play key roles in host cell invasion. The completion
22 of the *P. falciparum* and *A. gambiae* genomes has provided information about important
23 molecular events involved in parasite-insect interactions. The knockout studies of many
24 genes expressed in the mosquito stages have given hints of their biological functions and
25 survival strategies of the parasite in mosquito gut. The three-dimensional structural

1 analysis of parasite surface proteins will be indispensable in understanding the structure-
2 function relationship that will contribute to the development of therapeutic and vaccine
3 strategies against malaria.

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FIGURE LEGENDS

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4 **Fig. 1.** The Fab of the transmission-blocking monoclonal antibody 2A8 bound by its
5 heavy chain (H, blue) to the B loop (B, green) of domain 2 of Pvs25 (D2, green). The
6 light chain of 2A8 Fab (L, grey) does not contact Pvs25, appearing to play no role in
7 direct binding (41). The Pvs25 triangular prism is formed by domain 1 (D1, cyan),
8 domain 2 (D2, green), domain 3 (D3, red) and domain 4 (gold), (PDB accession code:
9 1Z3G).

10

11 **Fig. 2.** The observed sheets of Pvs25 molecules in the Pvs25 crystal. The reference
12 molecule (red) contacts four symmetry mates (cyan) and two molecules related by crystal
13 lattice translations (blue). The six Pvs25 molecules (cyan) have the same triangular face
14 'up', where three other molecules (red, blue) have the opposite face 'up' (41). The edge I
15 of Pvs25 pack against edge I of the neighboring ribbon, edge II to edge III, and edge III
16 to edge II to form sheets in crystal, (PDB accession code: 1Z1Y).

17

18



