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 (29). It is estimated that 300 to 500 million cases of malaria and over 1 million deaths from the disease occur each year (49).

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 transmission. Development of the malaria parasite in the mosquito begins when the gametocyte forms of the parasite are picked up by the mosquito in the blood meal from an infected human and quickly develop into extracellular gametes in the mosquito midgut. After fertilization, round zygotes form and transform into banana-shaped ookinetes. The ookinetes are motile and must exit the gut by crossing the peritrophic membrane and midgut epithelium. On the basal side of the epithelium, surviving ookinetes lodge against the basal lamina and transform into spherical oocysts. In the oocyst, the parasite develops into several thousand sporozoites, which then exit the oocyst and are carried by the hemolymph to the mosquito’s salivary glands to infect another host (22).

There is ongoing research to develop antiparasite vaccines against each stage of the complicated life cycle of *Plasmodium* (17, 24). Liver-stage vaccines are intended to reduce infection rates, and asexual-blood-stage vaccines will reduce disease severity and the risk of death during infection. Transmission-blocking vaccines would prevent the spread of disease by targeting antigens expressed in the mosquito stage on the surfaces of the gametocyte, gamete, zygote, and ookinete forms of the parasite (6, 61). These vaccines induce antibodies in the human host that inhibit parasite development in the mosquito midgut and thereby block parasite transmission to another person.

This article reviews the biology and structural knowledge of the *Plasmodium* P25 and P28 proteins and their contributions to transmission-blocking vaccine development.
hesive protein (SOAP), which contains two unique cysteine-rich domains and interacts with laminin (13). Ookinetes that were deficient in SOAP exhibited significantly reduced midgut invasion and oocyst formation (13).

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

Several transmission-blocking vaccine formulations are being developed using Plasmodium vivax and P. falciparum P25 and P28 proteins produced in Saccharomyces cerevisiae. Antisera from mice immunized with recombinant Pvs25 (P25 from P. vivax parasites) completely prevented the appearance of oocysts in mosquitoes that had ingested the antisera with P. vivax parasites (21). In a phase I vaccine trial of Pvs25 bound to aluminum hydroxide, the levels of antibodies that were generated correlated with transmission-blocking activity (34). Antibodies obtained after immunization of mice and monkeys with yeast-produced Pfs25 (P25 from P. falciparum parasites) (73) showed significant transmission-blocking activity in experiments in which a mixture of antisera, blood, and parasite cultures were fed to the mosquitoes through a membrane feeding apparatus (22). In humans, priming with a PfS25 gene-containing vaccinia virus and boosting with Pfs25 protein yielded antisera with significant transmission-blocking activity (25). Covalent conjugation of P25 proteins by chemical cross-linking to carrier proteins is a promising strategy, as it yields strong and sustained antibody responses (31, 37, 67).

INTERACTIONS OF P25 AND P28 WITH MOSQUITO MIDGUT PROTEINS

The interaction of ookinetes with the basal lamina is important for ookinete invasion and oocyst development in the mosquito. Plasmodium P25 and P28 proteins play an important role in parasite recognition of and attachment to the mosquito midgut (45, 46, 56). The Plasmodium berghei P25 and P28 proteins were shown, by yeast two-hybrid experiments, to interact with laminin, a major constituent of the basal lamina surrounding the midgut of Anopheles gambiae mosquitoes (64). The Plasmodium gallinaceum P25 and P28 proteins interact with the midgut basement membrane in order to attach the parasite to its surface (1). The P25 protein of P. berghei binds to laminin and collagen IV, and the binding is involved in the transformation of ookinetes into oocysts (3).

A study that combined knowledge of the sequenced genomes of Drosophila melanogaster and A. gambiae identified annexin proteins, which bind to P. berghei ookinetes during invasion of the mosquito midgut and play important roles in mosquito infection (30). When a blood meal containing a mixture of P. berghei parasites and anti-annexin serum was fed to mosquitoes in membrane feeding experiments, the number of observed oocysts was considerably reduced compared to that of the control. Confocal analysis of dissected midguts with anti-anopheles annexin mouse serum and the 13.1 MAb recognizing P. berghei P28 revealed that the staining of P28 and annexin overlapped and so the two proteins colocalized (30).

GENE DISRUPTION STUDIES

Gene disruptions of P25 and P28 revealed that the two proteins have partially redundant functions in parasites and are involved in ookinete survival in the midgut, penetration of the midgut epithelium, and the transformation of ookinetes to oocysts (56). When blood infected with P. berghei having either the P25 or the P28 gene disrupted was fed to mosquitoes through a membrane feeder, oocyst formation was slightly affected compared to oocyst formation in mosquitoes infected with wild-type P. berghei. However, when both genes were knocked out at the same time, almost no oocysts were formed (56). The ookinetes of the double-knockout parasite were swollen in appearance and did not cluster together in the gut as wild-type parasites do (56). A recent study of midgut epithelium invasion by double-knockout P. berghei ookinetes implied that the loss of P25/P28 proteins greatly reduced, but did not entirely prevent, the entry of ookinetes into midgut epithelial cells (5).

STRUCTURAL STUDIES

Primary structure. The 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 glycosylphosphatidylinositol moiety that anchors the proteins to the parasite surface (24, 27). Sequence analysis shows that P25 proteins contain 22 cysteine residues held together with 11 disulfide bonds and that P28 proteins contain 20 cysteine residues with 10 disulfide bonds. EGF-like domains are found predominantly in extracellular proteins of eukaryotes, where they participate in adhesion and signaling (2). A typical EGF-like domain contains 40 to 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 a 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 that used for vaccine trials (34, 42). The Pvs25 structure 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 interdomain contacts within the molecule and intermolecular contacts involved in sheet formation in the crystal are highly conserved in P25 and P28 proteins from all Plasmodium species (42).

Examination of the P. falciparum P25 sequence revealed that the Pfs25 protein likely assumes the same triangular structure as Pvs25. There is complete conservation of the residues forming the contacts among EGF-like domains 1, 3, and 4 that bring the four domains into their shape. The overall sequence identity between Pfs25 and Pvs25 is 46%, yet Pfs25 has been predicted to be similar to Pvs25 due to the disulfide-bonding similarities of the EGF-like domains. Pvs25 and Pfs25 are related, exhibiting 41% amino acid sequence identity over 157
residues of Pvs28. The residues that interact between domain 1 and domains 3 and 4 in Pvs25 are conserved throughout the sequences of the P28 family. Therefore, the structure of Pvs25 will likely be a valid model for the structures of all P28 family members (42).

**Sequence polymorphisms.** There are relatively few sequence polymorphisms in P25 and P28 proteins isolated from *P. falciparum* and *P. vivax* populations in the field, presumably because the P25 and P28 proteins are not expressed in the vertebrate host and thus are not exposed to selection pressure from the vertebrate immune system (8). In Pfs25, two conserved amino acid substitutions and two silent changes were found, while in the Pfs28 protein a Lys-to-Arg change at position 72 had been found and, recently, a new nonsynonymous polymorphism in P25 and P28 proteins isolated from *P. falciparum* and *P. vivax* members (42).

The Pvs25 crystal structure revealed a triangular prism-shaped structure that could tile the parasite surface (42). In the crystal, Pvs25 is packed as tightly arranged sheets that could also occur on the parasite surface to form a protective coat in the midgut (16). When a mosquito takes a blood meal from an infected person, the P25 and P28 proteins are expressed early and coat the parasite surface. Interactions between P25/P28-coated cell surfaces (46) may mediate parasite clustering, as these proteins are extremely abundant on ookinete surfaces (48). P25/P28 double-knockout and antibody-treated ookinetes did not cluster together (46, 47, 56) in the blood meal as do wild-type and untreated ones (68). Lack of clustering arising from lessened interactions between adjacent parasites may expose even the inner ookinetes of the cluster to the damaging proteolytic conditions of the midgut (16).

The Pvs25 crystal structure revealed a triangular prism-shaped structure that could tile the parasite surface (42). In the crystal, Pvs25 is packed as tightly arranged sheets that could also occur on the parasite surface to form a protective coat (Fig. 2). P25 and P28 can substitute for each other in single-knockout parasites (56). Perhaps either one alone can form an effective surface sheet, but a more protective one might be formed when both proteins are present. Yeast two-hybrid experiments (46) showed that Pvs25 has a tendency to form dimers, a requirement for forming a sheet.

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

**Involvement with ookinete entry into midgut epithelial cells.** The migration of P25/P28 double-knockout parasites into and

![FIG. 1. Fab of the transmission-blocking MAb 2A8 bound by its heavy chain (H; blue) to the B loop (B; green) of domain 2 of Pvs25 (D2; green). The light chain of 2A8 Fab (L; gray) does not contact Pvs25, appearing to play no role in direct binding. The Pvs25 triangular prism is formed by domain 1 (D1; cyan), domain 2 (D2; green), domain 3 (D3; red), and domain 4 (D4; gold) (PDB accession code 1Z3G). (Reprinted from reference 42 with permission of the publisher).](image_url)
abolished, compared to that of wild-type parasites (56). A
across the midgut epithelium is significantly reduced, but not
with permission of the publisher).
crystal (PDB accession code 1Z1Y). (Reprinted from reference 42
against edge III, and edge III packs against edge II to form sheets in
Pvs25 packs against edge I of the neighboring ribbon, edge II packs
Pvs25 molecules (cyan) have the same triangular face up, where three
and two molecules related by crystal lattice translations (blue). The six
The reference molecule (red) contacts four symmetry mates (cyan)
invasion by wild-type
33) and particular serpin molecules (10, 11). Midgut cells that
lumen, and increased expression of nitric oxide synthase (20,
during ookinete-to-oocyst development. The structure of
Gene knockouts show that P25 and P28 share multiple func-
tions during ookinete-to-oocyst development. The structure of
Pvs25 from P. falciparum
spp.;
Plasmodium
A. gambiae
isolates. This feature simplified the vaccine design, as a vaccine
based on a single target gene sequence will be effective for all
parasite isolates in various geographic locations. On the other
hand, a lack of expression in the human host indicated that this
vaccine would not be boosted by natural malaria infection. As
a result, the vaccine has to be highly potent to induce adequate
antibody levels that are sustained for at least one transmission
season. Vaccination of mice, rabbits, and monkeys with yeast-
produced Pfs25, with aluminum hydroxide as an adjuvant, in-
duces transmission-blocking antibodies (4, 26, 38). Yeast-pro-
duced Pvs25 formulated with aluminum hydroxide as an
adjuvant was evaluated in a phase I clinical trial (34). However,
antibody titers produced after Pvs25 immunization are low,
and more potent adjuvant formulations are required for induc-
ing high antibody titers. Yeast-produced Pfs25 formulated with
Montanide ISA51 is in a phase I clinical trial. Despite the
evidence of Pfs25 being an effective vaccine target, the protein
requires potent adjuvant formulations to increase the immu-
nogenicity to sustain high antibody titers. A covalent chemical
conjugate of yeast-produced Pfs25 linked to OMPC (the outer
membrane protein complex of Neisseria meningitidis serogroup
B) and formulated with aluminum hydroxyphosphate was
more effective in generating anti-Pfs25 enzyme-linked immu-
nosorbent assay reactivity than Pfs25 alone in Montanide
ISA720 at the same dose (67). The conjugate vaccine Pfs25-
OMPC has shown sustained high immunogenicity in rhesus
monkeys (67). Conjugating Pfs25 to a nontoxic recombinant
Pseudomonas aeruginosa exoprotein A (37) or to Pfs25 itself to
form multimeric molecules (31) also significantly increased the
immunogenicity of Pfs25.

CONCLUDING REMARKS

Vaccines targeting the Plasmodium P25 and P28 proteins are
promising strategies for malaria control, as they induce human
antibodies that inhibit the parasite in the mosquito midgut.
Gene knockouts show that P25 and P28 share multiple func-
tions during ookinete-to-oocyst development. The structure of
Pvs25 from P. berghii is the first of a mosquito-stage surface
protein and has a novel arrangement of EGF domains. Pvs25
forms triangular prism structures, and residues forming the
triangles are highly conserved in all other Plasmodium spp.;
thus, Pvs25 is a good template for predicting the structures of
other P25 and P28 proteins. P25 and P28 interactions with
transmission-blocking antibodies indicate that antibodies bind
to the B loops of the second and third EGF-like domains of
P25 and P28 proteins. The complex of Pvs25 with a Fab frag-
ment showed how one transmission-blocking antibody binds
P25; the generation of such antibodies by inexpensive and
simple transmission-blocking vaccines should play an impor-
tant role in the control of malaria transmission.

Plasmodium surface proteins play key roles in host cell in-
vasion. The completion of the P. falciparum and A. gambiae
genomes has provided information about important molecular
events involved in parasite-insect interactions. Knockout stud-
ies of many genes expressed in the mosquito stages have given
hints of their biological functions and survival strategies of the
parasite in the mosquito gut. The three-dimensional structural
analysis of parasite surface proteins will be indispensable in
understanding the structure-function relationship that will con-

VACCINE DEVELOPMENT

Transmission-blocking vaccines against the two major spe-
cies of human malaria parasite, P. falciparum and P. vivax, are
under development (6, 61). Both P25 and P28 are encoded by
single-copy genes that are highly conserved among parasite

FIG. 2. Observed sheets of Pvs25 molecules in the Pvs25 crystal. The reference molecule (red) contacts four symmetry mates (cyan) and two molecules related by crystal lattice translations (blue). The six Pvs25 molecules (cyan) have the same triangular face up, where three other molecules (red and blue) have the opposite face up. Edge I of Pvs25 packs against edge I of the neighboring ribbon, edge II packs against edge III, and edge III packs against edge II to form sheets in crystal (PDB accession code 1Z1Y). (Reprinted from reference 42 with permission of the publisher).
to contribute to the development of therapeutic and vaccine strategies against malaria.

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