phenotype (*14*), although it did not increase the risk of chloroquine treatment failures in association with *pfcrt* mutations (*8*). Mutations in the *pfmdr1* gene may therefore represent fitness adaptations to physiological changes from the *pfcrt* mutations, analogous to the compensatory alterations that occur in other pathogens after the acquisition of core resistance determinants (*15*). Effects from possible modulators of CQR phenotypes may vary with the genetic background of *P. falciparum* in different geographical regions. If so, methods that dissect quantitative trait loci in genetic crosses can be applied to map chromosome segments and search the database for genes involved in these effects. Determinants of more complex forms of drug resistance (e.g., those responsible for decreases in quinine efficacy in South America and Southeast Asia) may also be identifiable through these approaches.

Searches for new drugs to meet the need once filled by chloroquine require targets that are parasite specific and can be hit with prompt and lethal effect. Drugs identified by these searches, however, may encounter forms of resistance from one or two target mutations and soon lose their reliability for treating cases of *P. falciparum* malaria. Examples of such target mutations include parasite dihydrofolate reductase mutations that counter the action of pyrimethamine and cytochrome b mutations that counteract atovaquone; both alterations quickly produce resistance under drug pressure. Pyrimethamine and atovaquone have consequently been formulated with different target inhibitors such as sulfadoxine (Fansidar combination) and proguanil (Malarone combination), respectively. Combinations of inhibitors that hit the same active site but exert opposing selective forces on particular target mutations may also offer an effective strategy to slow the spread of resistant strains (*16*). New targets in metabolic pathways mined from the *P. falciparum* genome sequence, although subject to these considerations, provide some exciting prospects for drug searches. Among these are targets in the type II fatty acid and the mevalonate-independent (1-deoxy-D-xylulose-5-phosphate or DOXP) isoprenoid pathways of the parasite apicoplast, a chloroplast-like organelle (*17, 18*). Already, fosmidomycin—an inhibitor of DOXP reductoisomerase evaluated previously for its antibacterial activity—has been found to have significant antimalarial activity and is currently being tested in a synergistic combination with clindamycin (*19*).

Among old and new targets for antimalarials, the host heme molecule attacked by chloroquine remains one of the most attractive for drug development. Antimalarials that act on heme or take advantage of its potential for oxy radical activation can produce prompt clearances of parasites from the bloodstream. Important examples in addition to chloroquine include the quinine alkaloids and endoperoxide-containing artemisinin derivatives used in the treatment of severe malaria. The mechanism of CQR in *P. falciparum* probably involves specific structural interactions between chloroquine and amino acid substitutions in PfCRT that were slow to evolve because of their complexity. This could be good news for drugs that attack heme detoxification but are not recognized by the CQR mechanism, as *P. falciparum* may again find it difficult to develop mutations required for resistance. Indeed, a number of 4-aminoquinoline analogs of chloroquine and other inhibitors of heme polymerization have been identified that are effective against both chloroquine-resistant and chloroquine-sensitive *P. falciparum* parasites (Fig. 1) (*20–22*). The favorable pharmaceutical properties of certain of these compounds have established them as promising leads for drug discovery, and some are being pursued in private-public partnering arrangements sponsored by organizations such as the Malaria Medicines Venture (www.mmv.org). Although the standards established by chloroquine are high and the process required to bring new drugs forward is expensive, the development of an affordable chloroquine replacement effective against CQR malaria will be an achievement that more than justifies the necessary effort.

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VIEWPOINT

# The *Plasmodium falciparum* Genome—a Blueprint for Erythrocyte Invasion

**Alan F. Cowman and Brendan S. Crabb**

**Erythrocyte invasion by *Plasmodium falciparum* involves multiple ligand-receptor interactions and numerous apparent redundancies. The genome sequence of this parasite reveals new gene families encoding proteins that appear to mediate erythrocyte invasion.**

The invasion of human red blood cells by the extracellular merozoite form of *Plasmodium falciparum* is a process central to the pathogenesis of this devastating pathogen. Human erythrocytes demonstrate remarkable diversity with regard to the surface molecules they express. Critical to the success of *P. falciparum* is the flexibility this parasite shows when attaching to and invading host red blood cells. Consequently, *P. falciparum* is able to invade erythrocytes that are antigenically different (as a result of age or allelic diversity) through a number of alternate pathways that involve distinct receptors. After attachment to its target host cell, the merozoite reorients itself such that its pointed (or apical) end becomes positioned at the site of entry (Fig. 1). Thus, the proteins of the merozoite surface, together with those of the organelles associated with the apical end, are considered vital to erythrocyte invasion. It is clear from the recently completed *P. falciparum* genome sequence that many of the molecules thought to be involved in invasion are members of larger gene families (*1, 2*). Although much remains to be learned, the specific involvement of these molecules in determining independent invasion pathways, as well as their importance in the induction of antiparasite immunity, is becoming clearer. The availability of the *P. falciparum* genome has already provided a wealth of information that has greatly accelerated our understanding of the complex process of invasion.

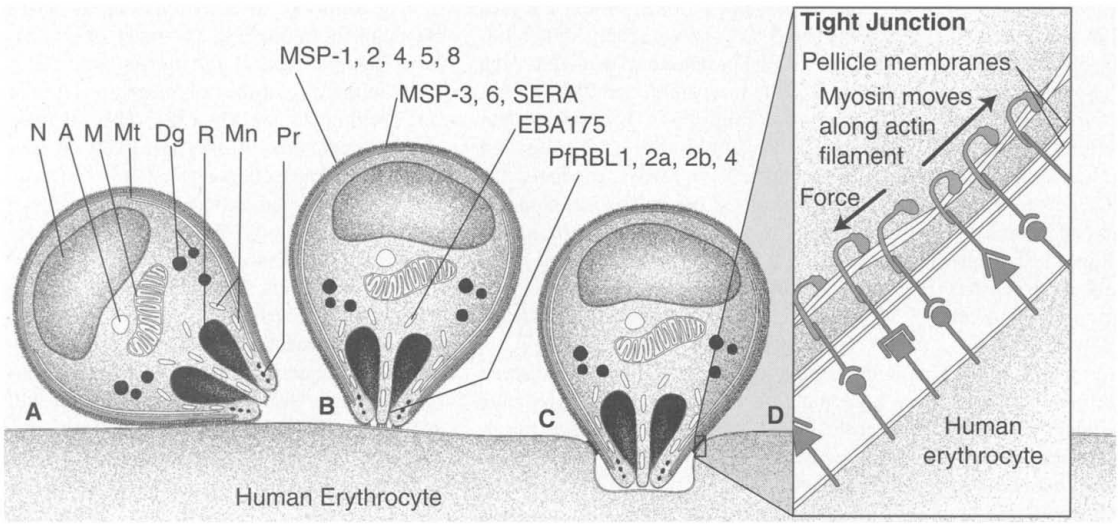


**Fig. 1.** A model for merozoite invasion of the human erythrocyte. (**A**) Invasive extracellular merozoites of *P. falciparum* are released by rupture of the mature schizont form and bind to the erythrocyte surface in a low-affinity interaction. (**B**) The bound merozoite then undergoes reorientation so that its apical end comes into close contact with the erythrocyte surface. Proteins at the apical end that interact with the red blood cell are likely to include the PfRBL proteins, which may trigger release of the protein components of the micronemes onto the merozoite surface, where they interact with high-affinity erythrocyte receptors such as glycophorin A. This is important for development of a tight junction around the merozoite and for entry of the merozoite into the erythrocyte. (**C** and **D**) Merozoite proteins in the tight junction interact with receptors on the erythrocyte surface and are connected with actin-myosin motors on the cytoplasmic face of the parasite. Active movement of this tight junction across the merozoite surface allows the merozoite to invade the erythrocyte. During this process, cleavage of the merozoite surface proteins releases these proteins from the actin-myosin motors. The end result of the invasion process is internalization of the merozoite in the erythrocyte and eventual enclosure by the parasitophorous vacuole membrane. N, nucleus; A, apicoplast; M, mitochondrion; Dg, dense granules; R, rhoptry; Mn, micronemes; Mt, microtubule; Pr, polar rings; MSP, merozoite surface protein; EBA175, erythrocyte binding antigen 175; PfRBL, *P. falciparum* reticulocyte binding protein homologs; SERA, serine repeat antigen.

## Merozoite Surface Proteins (MSPs)

MSPs, [2]the integral membrane proteins identified on the surface of developing and free merozoites, are likely to be important for invasion (Fig. 1); antibodies directed against these proteins block invasion. All membrane-associated MSPs have a glycosylphosphatidylinositol (GPI) membrane anchor attached to the COOH-terminus. Of the five GPI-linked MSPs (MSP-1, 2, 4, 5, and 8) previously identified in *P. falciparum*, all except MSP-2 have one or two epidermal growth factor (EGF)–like domains at their COOH-terminus. These domains are of particular interest because of their possible role in mediating key protein-protein interactions (by analogy with other EGF-like domains) and because they are clearly targeted by protective immune responses of the host. For instance, the double EGF domain found at the COOH-terminus of MSP-1, known as MSP-$1_{19}$, is the target of protective immunity [reviewed in (*3*)] involving probable production of invasion-inhibitory antibodies (*4*). As a result, recombinant forms of MSP-$1_{19}$ have become leading vaccine candidates. Examination of the *P. falciparum* genome database for additional EGF-like domain–containing molecules has already led to the identification of molecules implicated in erythrocyte invasion. One such molecule is MSP-8, which, like MSP-1, has a double EGF domain at its COOH-terminus (*5*). Additionally, another gene encoding a double EGF–containing membrane protein with considerable similarity to MSP-1 and MSP-8 has been identified (*6*). The recent identification of MSPs with MSP-$1_{19}$–like domains underscores the likely importance of this double EGF module in invasion and has implications for vaccine development. For example, it is feasible that antibodies targeting more than one of these double EGF modules may act synergistically to inhibit merozoite invasion, enhancing the protective effect.

The organization of genes implicated in erythrocyte invasion may also provide insight into the evolution of the repertoire of molecules implicated in invasion, as well as their possible function. The *MSP-4* and *MSP-5* genes (encoding the related single EGF–containing proteins MSP-4 and MSP-5) are found adjacent to one another on chromosome 2, immediately upstream of *MSP-2* (*7*), and probably arose through gene duplication. However, in other *Plasmodium* species, this head-to-tail array of the *MSP-4*, *MSP-5*, and *MSP-2* genes is replaced in the syntenic region either by a single gene showing homology to both *MSP-4* and *MSP-5* (*8*) or by two *MSP-4/5*–like genes (*5*). Hence, it seems that either *P. falciparum* MSP-2 performs a unique function not required in all *Plasmodia*, or its role can be performed by other genes, perhaps by *MSP-4/5* homologs, in other species.

Already the advent of the genome project has facilitated the rapid identification of genes (and gene families) encoding invasion-associated molecules characterized functionally. For instance, MSP-1 is part of a noncovalently bound complex at the merozoite surface containing two other molecules, MSP-6 and MSP-7 (*9, 10*). After $NH_2$-terminal sequencing of the polypeptides of the complex, the genes encoding both MSP-6 and MSP-7 have been identified using the genome sequence (*10, 11*). MSP-6 is related to MSP-3, a molecule also implicated in invasion, and both of these genes reside in a cluster of 12 genes on chromosome 10 (*1*). MSP-3 is a polymorphic protein that binds to the merozoite surface, after trafficking to the parasitophorous vacuole (*12*), and is the target for host antibodies that inhibit *P. falciparum* growth in vitro. Truncation of this protein by gene targeting suggests that it is involved in merozoite invasion, because the mutant merozoites invade human erythrocytes less efficiently than normal merozoites (*13*). Located adjacent to *MSP-3* and *MSP-6* are genes encoding a glutamic acid–rich protein (GLURP) (*14*) and S-antigen (S-Ag) (*15*), both of which are localized to the parasitophorous vacuole and possibly also at the merozoite surface. Surrounding this cluster of four genes are another eight genes, all of which have properties of secreted proteins similar to MSP-3/6, GLURP, and S-Ag as well as having some shared motifs. It is tempting to speculate that the proteins encoded by this cluster of genes arose by a complex series of gene duplication events and diverged to provide the parasite with related but distinct functions.

Like MSP-3, the serine repeat antigen (SERA) is also localized to the parasitophorous vacuole and merozoite surface. From genomic sequencing, it now appears that the

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*SERA* gene (known as *SERA5*) is just one of eight *SERA*-like genes found in a head-to-tail array on chromosome 2 (*1*, 2). Another *SERA* homolog has been identified on chromosome 9 (*1*, *16*). It has been known for some time that SERA is abundant in mature-stage parasites and that its $NH_2$-terminal domain is the target of antibodies that effectively inhibit invasion. The presence of relatively large *SERA* multigene families in *P. falciparum* and in other divergent *Plasmodium* species suggests an important biological role for the proteins encoded by these genes (*16*). All SERAs have related protease domains, and hence the possibility remains that there is functional redundancy among the family members and that their expression pattern may change in response to immune pressure and/or red blood cell diversity.

## Apical Organellar Proteins

Apical binding of the merozoite initiates development of a tight junction between the merozoite and erythrocyte surface. The ability of *P. falciparum* to use alternate human erythrocyte receptors during merozoite invasion implicates multiple parasite ligands in these processes (*17*). Indeed, *P. falciparum* has more than four alternate invasion pathways. In contrast, malaria parasites such as *P. vivax* appear to depend on interactions with a single receptor on human erythrocytes (the Duffy blood group antigen). A number of protein families have been identified in other *Plasmodia* species that may be involved in invasion, and it is likely that homologs of these proteins are present in *P. falciparum*. The availability of the *P. falciparum* genome sequence allows testing of this hypothesis.

A family of proteins from the rodent malaria *P. yoelii* (the Py235 family) have been implicated in the ability of this parasite to invade mature mouse erythrocytes (*18*). Related reticulocyte binding proteins have been identified in *P. vivax* (PvRBP-1 and PvRBP-2). There is suggestive evidence that these proteins are involved in the apical interaction of the merozoite with the erythrocyte surface (*19*). Using the *P. falciparum* genome database, four genes (*PfRBL1*, *PfRBL2a*, *PfRBL2b*, and *PfRBL4*) have been identified that share homology and structural features with the Py235 and PvRBP protein families (*20, 21*). The *P. falciparum* reticulocyte binding–like (PfRBL) proteins are localized at the apical end of the merozoite, and PfRBL1 has been shown to bind to a trypsin-resistant receptor on the erythrocyte (*21*). We still need to elucidate how PfRBL mediates the apical interaction of the merozoite with the human erythrocyte, and to clarify the mechanisms of tight junction formation and invasion via different receptor pathways.

The erythrocyte binding antigen EBA175 of *P. falciparum* binds to glycophorin A on the erythrocyte surface and may be involved in tight junction formation at the merozoite surface. This parasite ligand mediates an alternate invasion pathway via interactions with glycophorin A (*22*). EBA175 is a member of a larger protein family that contains structural motifs required for specific recognition of host receptors. Database mining of the *P. falciparum* genome has identified three genes with close homology to *EBA175* (*23*). The proteins encoded by these genes have the same structural motifs, which suggests that they are adhesive proteins involved in merozoite invasion. Because EBA140 (BAEBL) binds to the erythrocyte surface, using a receptor distinct from glycophorin A, it may be important for merozoite invasion via a direct ligand-receptor interaction (*24, 25*). The identification of likely paralogs of EBA175 has prompted further study of the role of this protein family in the ability of this parasite to invade erythrocytes by means of multiple receptors.

An interesting feature apparent from the *P. falciparum* genome sequence is that *EBA175* and its related homologs are dispersed on different chromosomes and located near the subtelomeric regions (*1*). The ends of each chromosome have a conserved structure consisting of repeat regions that favor pairing of heterologous chromosomes and ectopic recombination (*26*). This would provide a mechanism for the generation of new forms of genes located toward the ends of chromosomes and the development of gene families. It is likely that the *EBA* gene family arose from a single precursor by recombination between heterologous chromosomes and then diverged to provide parasite ligands with different host receptor specificities.

The ability to invade human erythrocytes by means of multiple ligand-receptor interactions has provided *P. falciparum* with flexibility in the face of the polymorphic nature of the erythrocyte surface (*22*). The alternate invasion pathways it uses are likely to provide added protection against host immune responses mounted against parasite ligands of a particular pathway. The availability of the *P. falciparum* genome sequence (*1*) provides enormous impetus for the functional characterization of the parasite proteins involved in merozoite invasion, and this will greatly increase our understanding of host-parasite interactions. Perhaps the greatest challenge will be to use this information efficiently to determine the most suitable therapeutic targets for developing and testing new vaccines and drugs.

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