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The cell biology of disease

The cellular and molecular basis for malaria parasite invasion of the human red blood cell

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Malaria is a major disease of humans caused by protozoan parasites from the genus *Plasmodium*. It has a complex life cycle; however, asexual parasite infection within the blood stream is responsible for all disease pathology. This stage is initiated when merozoites, the free invasive blood-stage form, invade circulating erythrocytes. Although invasion is rapid, it is the only time of the life cycle when the parasite is directly exposed to the host immune system. Significant effort has, therefore, focused on identifying the proteins involved and understanding the underlying mechanisms behind merozoite invasion into the protected niche inside the human erythrocyte.

Introduction

Five species of *Plasmodium* parasite cause malaria, and there is growing awareness of the importance of each to global health (World Health Organization, 2010). The majority of mortality and morbidity attributed to malaria are caused by *Plasmodium falciparum* (Snow et al., 2005); however, *Plasmodium vivax* also causes a significant burden of disease (Guerra et al., 2010). Infection by all *Plasmodium* spp. begins with the bite of an infected female *Anopheles* mosquito (Fig. 1). After a silent infectious phase, primarily in the liver hepatocyte (Prudêncio et al., 2011), exoerythrocytic merozoite forms are passed into the blood stream as membrane-bound merosomes that rupture, allowing parasites access to circulating erythrocytes (Fig. 1; Sturm et al., 2006; Prudêncio et al., 2011). The merozoites rapidly invade erythrocytes, and as they grow and replicate, the intracellular parasite dramatically remodels the host red blood cell, giving rise to a rigid and poorly deformable cell with a propensity to adhere to a variety of cell types. These changes play a pivotal role in severe complications of *P. falciparum* malaria, with symptoms including fever, anemia (though not necessarily resulting from loss of blood cells; Evans et al., 2006), lactic acidosis, and in some cases coma and death (for review see Miller et al., 2002).

Clinical immunity to malaria is slow to develop and short lived. One reason for this is the extensive diversity found in *Plasmodium* antigens, which facilitate parasite escape from host immune detection. This antigenic diversity in *P. falciparum* arises by two main mechanisms. Classical antigenic variation allows a clonal lineage of *P. falciparum* to express successive alternate forms of a variant antigen on the surface of the infected-erythrocyte (for review see Kirkman and Deitsch, 2012). There is also a large amount of antigenic diversity created by allelic polymorphisms, most of which likely arose from host immune selection. The merozoite also displays a form of phenotypic variation in which different strains express a variant combination of functional ligands that bind to specific receptors on the erythrocyte (Duraisingh et al., 2003; Stubbs et al., 2005). This provides a mechanism to escape host immune detection and to counteract the polymorphic nature of the erythrocyte surface, much of which has been driven by parasite evolutionary pressure. An example is the preponderance of Duffy antigen/chemokine receptor (DARC) negativity in West African populations. *P. vivax* is generally unable to invade Duffy-negative erythrocytes, and this variant therefore protects the population from this species (Miller et al., 1976). Recent work has, however, identified *P. vivax* parasites in Madagascar that invade Duffy-negative erythrocytes, which suggests that DARC-independent host cell invasion is possible (Ménard et al., 2010). The mechanisms of antigenic and phenotypic diversity developed by the malaria parasite and the genetic polymorphisms in the human population linked to protection against this disease are an indication of a long-running genetic war between pathogen and host.

A case can be made for a vaccine targeting each stage of parasite development (Fig. 1); however, the blood stage specifically has been a longstanding focus for vaccine efforts.

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Abbreviations used in this paper: DBL, Duffy binding–like; EBL, erythrocyte binding–like; GPI, glycosylphosphatidylinositol; IMC, inner membrane complex; MTRAP, merozoite TRAP; PTRAMP, *Plasmodium* thrombospondin-related apical merozoite protein; SERA, serine repeat antigen; TRAP, thrombospondin-related anonymous protein.

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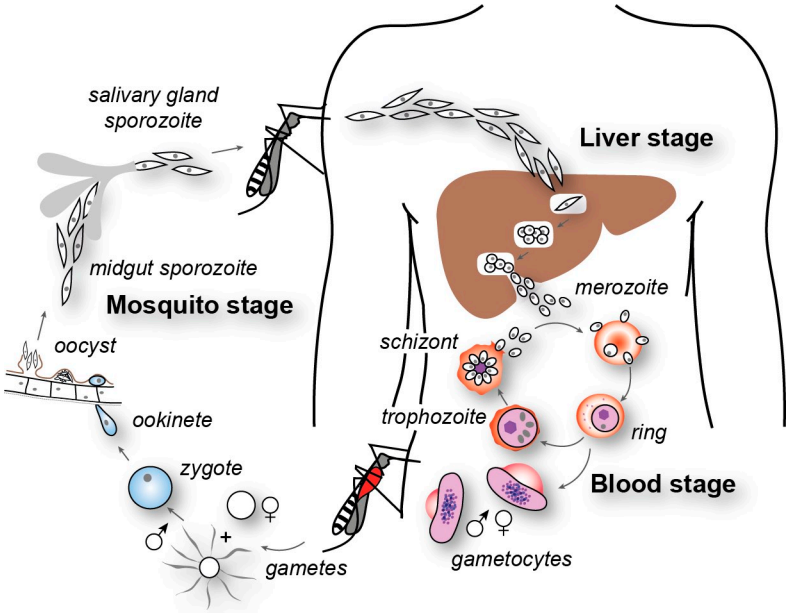

Figure 1. **The life cycle of *P. falciparum*.** The *Anopheles* mosquito bites a human and injects sporozoite forms. These move to the liver and invade hepatocytes, in which they develop to produce exoerythrocytic merozoite forms that are released into the blood stream. Merozoites invade erythrocytes and grow into trophozoites and mature schizonts. Merozoites are released that reinvade new erythrocytes. Gametocytes, formed from the asexual blood stage, are taken up by a feeding mosquito into the gut where they mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands.

Underlying this rationale, in addition to its central role in disease pathology, is strong evidence that merozoite antigens are targets of protective immunity (Cohen and Butcher, 1970; Persson et al., 2008) and of the ability of antibodies targeting these proteins to block erythrocyte invasion (Wåhlin et al., 1984; Blackman et al., 1994; Lopaticki et al., 2011). However, to date, efforts to generate an effective blood stage vaccine have not met with much success primarily because of antigenic diversity and a poor understanding of protective host immune responses (for review see Anders et al., 2010). In recent years, developments in genomics and systems approaches have increased understanding of merozoite proteins involved in host cell invasion as well as host immune responses (Cowman and Crabb, 2006; for review see Anders et al., 2010), which lies at the core of recent strategies to develop blood stage vaccines to aid future efforts to control this global disease.

Merozoite biology

The blood stage merozoite is the smallest cell within the *Plasmodium* lifecycle. Indeed, it is one of the smallest eukaryotic cells known (~1–2 µm) and is exquisitely adapted for invasion of erythrocytes (Bannister et al., 1986). The merozoite has the conventional organelle repertoire of eukaryotic cells with the overall cytoskeletal architecture of an apicomplexan cell (Morrissette and Sibley, 2002), the phylum to which malaria parasites belong (Fig. 2 A). This includes an apical complex of secretory organelles (micronemes, rhoptries, and dense granules), mitochondrion, nucleus, and relict plastid (apicoplast; McFadden et al., 1996; Roos et al., 1999; Bannister et al., 2000b). Underlying the plasma membrane is a membranous network of flattened vesicles called the inner membrane complex (IMC), which is subtended by two to three subpellicular microtubules (for review see Bannister et al., 2000a). In recent years, definition of the apical secretory organelles has blurred with the identification of dense granule-like exonemes (associated with parasite egress; Singh et al., 2007). As the molecular definition of these and other compartments expands, refinement of the identity and naming of organelles will be required.

A cellular overview of invasion

The cellular steps of invasion have been studied by microscopy in both *P. falciparum* and *Plasmodium knowlesi* (Dvorak et al., 1975; Glushakova et al., 2005; Gilson and Crabb, 2009). Initially, the mature merozoites are propelled from the bursting schizont (the mature blood stage form) at egress (Glushakova et al., 2005; Abkarian et al., 2011), after which they associate with erythrocytes (Figs. 2 and 3). Initial interaction involves dramatic movement of the merozoite and deformation of the erythrocyte surface followed by a seemingly active process of reorientation that places the parasite apex abutting the host cell membrane. After a brief pause and major buckling of the erythrocyte surface, possibly as a result of parasite-induced reorganization of the erythrocyte cytoskeleton (Zuccala and Baum, 2011), the parasite enters the erythrocyte (Fig. 2 B). Sealing at the posterior of invasion is followed by a brief period of echinocytosis of the red cell (a morphological spiking of the cell stimulated by efflux of potassium and chloride ions), with the erythrocyte returning to its normal shape within 10 min (Gilson and Crabb, 2009). The internalized parasite, now referred to as a ring, undergoes rapid and dramatic changes in shape after this process (Grüring et al., 2011).

Much of the invasion process itself is organized around a key interface that forms between the two cells called the tight or moving junction, an area of electron density (by electron microscopy) and close apposition between the two cells (Fig. 2 B; Aikawa et al., 1978). This structure appears to coordinate distinct stages after egress and attachment, facilitating invasion and postinvasion sealing of the parasite within the erythrocyte (Fig. 3). However, although each step of

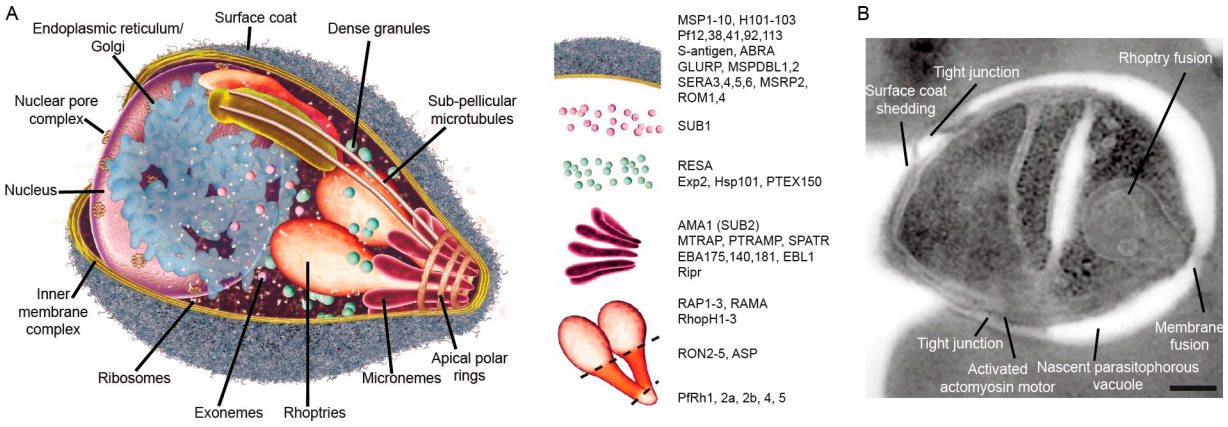

Figure 2. **Three-dimensional diagram of a merozoite and its core secretory organelles.** (A) The sectioned cell highlights the major cellular architecture and organelle repertoire of the invasive merozoite, with dissected organelles listing core molecular constituents of these key invasion-related compartments. Of note, though definition of secretory organelles is limited to dense granules, micronemes, and rhoptries, there is mounting evidence that subpopulations of organelles and subcompartmentalization within organelles (specifically the rhoptries) certainly exist. The rhoptries are divided into three segments, with PfRh1, -2a, -2b, -4, and -5 in the most distal segment and RON2-5 in the next segment. This organization is predicted based on functionality and early release of the PfRh proteins onto the merozoite surface during invasion as opposed to the release of the RON protein complex, but it has not yet been demonstrated definitively (Riglar et al., 2011). The dense granules are released very soon after invasion and include components of a putative protein translocon that is inserted into the parasitophorous vacuole membrane. Ring-infected erythrocyte surface antigen (RESA) is released from dense granules and exported to the infected red blood cell. The body of the rhoptry bulb contains lipids and other proteins involved in forming the parasitophorous vacuole, including RAP1-3 and RAMA. (B) A *P. falciparum* merozoite in the process of invading a human red blood cell (image courtesy of S. Ralph, University of Melbourne, Melbourne, Australia). Bar, 200 nm.

invasion has been described in detail by microscopy, they are incompletely understood at the molecular level and only recently described in cellular detail for *P. falciparum* merozoites (unpublished data). Availability of the genome sequence from *P. falciparum* and other *Plasmodium* spp. together with proteomic and transcriptional information has, however, greatly assisted in the identification of proteins associated with the merozoite. This includes many located on the surface or within micronemes and rhoptries, likely to be some of the critical proteins that mediate the molecular basis of invasion (Table 1 and Fig. 2 A).

Molecules involved in initial erythrocyte contact

Proteins located on the merozoite surface have been of interest over the years because they are considered prime vaccine candidates, being directly exposed to host immune responses on merozoite release (Egan et al., 1996). These are divided into proteins anchored to the merozoite plasma membrane via a glycosylphosphatidylinositol (GPI) anchor and others associated by interaction with surface proteins (Fig. 2 A). These proteins are not evenly spread over the merozoite and some have apical concentrations, which is consistent with a direct role in

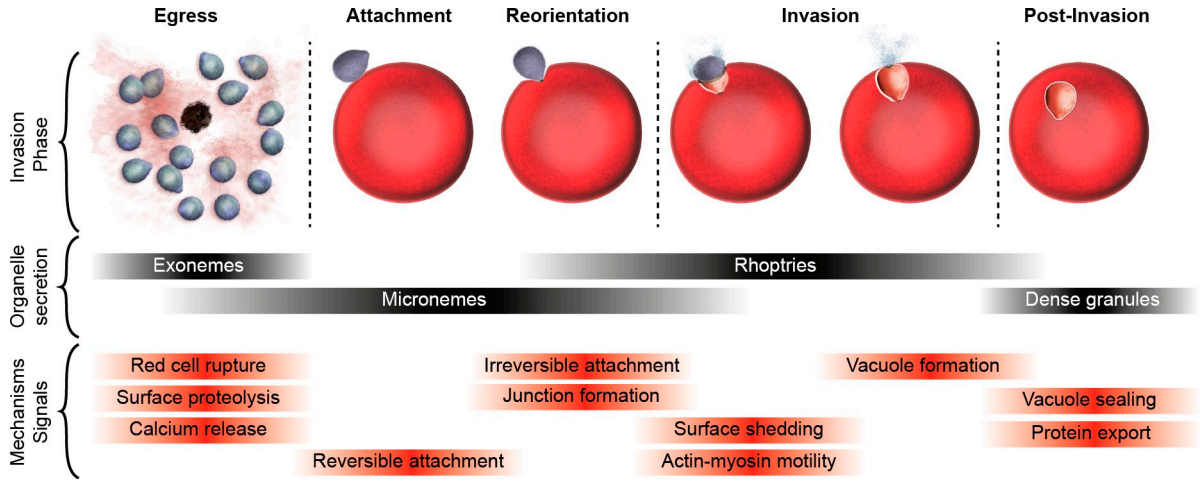

Figure 3. **A time course of merozoite invasion of the erythrocyte from egress through postinvasion.** (A) A cellular overview is given with associated timing of organelle secretion and key mechanistic or signaling steps listed below. After apical reorientation, the merozoite establishes a tight junction that is marked by RON4 and AMA1. The tight junction is ultimately connected to the actomyosin motor, although the exact nature of this has yet to be established. As the tight junction moves across the merozoite surface, proteins are shed into the supernatant through the activity of proteases such as ROM4, ROM1, SUB1, and SUB2. The parasitophorous vacuole and membrane are formed primarily from the rhoptries, although some red cell membrane components are included, which expel their contents, forming the space into which the parasite can move under the action of the actomyosin motor. Once the tight junction reaches the posterior end of the parasite, the membranes seal by an as yet unknown mechanism.

Table 1. **The invasion-related proteins of the *P. falciparum* merozoite**

Name	PlasmoDB accession number	Genetic knockout	Localization in merozoite before/during invasion	Potential function	Feature/structure
GPI-anchored MSPs					
MSP-1	PF3D7_0930300	N	Surface/complex shed during invasion with MSP1/19 EGF C-terminal domain retained in PV of ring stage	Putative Band 3 ligand; C-terminal double EGF domain redundant for divergent molecules: processed SUB1 and -2	Two C-terminal EGF domains: compact side by side arrangement
MSP-2	PF3D7_0206800	N	Surface	Highly polymorphic; likely structural role as surface coat	Unordered repetitive structure
MSP-5	PF3D7_0207000	N	Surface	Not known	C-terminal EGF domain
MSP-4	PF3D7_0206900.1	Y	Surface	Not known	C-terminal EGF domain
MSP-10	PF3D7_0620400	N	Surface	Not known	C-terminal EGF domain
Pf12	PF3D7_0612700	Y	Surface/shed	Potential adhesive protein	6-Cys domains
Pf38	PF3D7_0508000	Y	Surface/shed	Potential adhesive protein	6-Cys domains
Pf92	PF3D7_1364100	Y	Surface/shed	Not known	Cys-rich protein
Peripheral surface proteins					
Pf113	PF3D7_1420700	N	Surface/shed	Not known	No data
MSP-9 (ABRA)	PF3D7_1228600	Y	Surface/shed	Putative protease	No data
S-antigen	PF3D7_1035200	N	Secreted into PV of schizont and released on egress	Not known; potential immunomodulatory role	Highly repetitive and diverse protein
GLURP	PF3D7_1035300	Y	Secreted into PV of schizont and released on egress	Not known	Repetitive Glutamate-rich
MSP-3	PF3D7_1035400	Y	Surface/shed	Not known; binds to MSP-1	Repetitive and Glutamate-rich
MSP-6	PF3D7_1035500	Y	Surface/shed	Not known; binds to MSP-1	Leucine zipper-like C-terminal domain
H101 (MSP-11)	PF3D7_1035600	Y	Surface/shed	Not known	MSP-3 family, leucine zipper-like C-terminal domain
H103	PF3D7_1035900	Y	Surface/shed	Not known	MSP-3 family, leucine zipper-like C-terminal domain
MSP-7	PF3D7_1335100	Y	Surface/shed	Associates with MSP-1, gene knockout in *P. berghei* shows important in invasion of mature erythrocytes	No data
MSP-7-like (MSRP2)	PF3D7_1334800	Y	Surface/shed	Not known; may associate with MSP-1	MSP-7 family
MSPDBL-1	PF3D7_1036300	Y	Surface/shed	Binds to unknown receptor on red cell	Member of EBL family, DBL and leucine zipper-like domains
MSPDBL-2	PF3D7_1035700	Y	Surface/shed	Binds to unknown receptor on red cell	Member of EBL family, DBL and leucine zipper-like domains
SERA3	PF3D7_0207800	Y	Secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA4	PF3D7_0207700	N	Most secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA5	PF3D7_0207600	N	Secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA6	PF3D7_0207500	N	Most secreted into PV of schizont and released on egress	Cysteine protease domain with active site cysteine	Cysteine protease domain
Pf41	PF3D7_0404900	Y	Surface/shed	Potential adhesive protein; binds Pf12 on merozoite	6-Cys domains
Plasma membrane proteins					
ROM1	PF3D7_1114100	Y	Mononeme (proposed new apical organelle) or microneme/surface	Rhomboid protease; cleaves AMA1, MAEBL, EBLs, PfRh proteins; likely role after invasion in PV formation	Multipass transmembrane protein
ROM4	PF3D7_0506900	ND	Surface/shed	Rhomboid protease; cleaves AMA1, MTRAP, EBL, and PfRh proteins in transmembrane to allow shedding during invasion	Multipass transmembrane protein

Table 1. *(Continued)*

Name	PlasmoDB accession number	Genetic knockout	Localization in merozoite before/during invasion	Potential function	Feature/structure
Microneme proteins					
AMA 1	PF3D7_1133400	N	Micronemes/surface and binds to RON2 that has been inserted into red cell membrane and tracks with tight junction	Released on merozoite surface; binds RON complex; potential ligand for McLeod antigen, phosphorylation of cytoplasmic tail essential, may be involved in signaling	PAN (plasminogen, apple, nematode) motifs
EBA-175	PF3D7_0731500	Y[a]	Micronemes/surface and binds to glycophorin A	Binds to glycophorin A, likely signaling role for invasion	EBL family with DBL domains; "handshake" association between region II dimers creates groove for glycophorin A binding
EBA-181/JESEBL	PF3D7_0102500	Y	Micronemes/surface and binds to unknown receptor	Binds to unknown receptor on red cell	EBL family member with DBL domains
EBA-140/BAEBL	PF3D7_1301600	Y	Micronemes/surface and binds to glycophorin C	Binds to glycophorin C on red cell	EBL family member with DBL domains
EBL-1	PF3D7_1371600	Y	No data	Binds to glycophorin B, nonfunctional because of mutations causing truncated protein	EBL family member with DBL domains
PTRAMP	PF3D7_1218000	ND		Not known; cleaved by SUB2 on merozoite surface	Long extended structure
PfRipr	PF3D7_0323400	N	Micronemes/surface and binds to PfRh5	Binds to PfRh5	10 EGF domains, 87 cysteines
MTRAP	PF3D7_1028700	N	Micronemes/PV	Potential motor-associated protein	Thrombospondin-like domains
PTRAMP	PF3D7_1218000	N	Micronemes/surface	Potential motor-associated protein	Thrombospondin-like domains
SPATR	PF3D7_0405900	ND	Micronemes/surface	Not known for blood stages	Thrombospondin-like domains
GAMA	PF3D7_0828800	ND	Micronemes/surface	Binds to red cells; has GPI anchor	No data
SUB2	PF3D7_1136900	N	Micronemes/PV	Protease that processes MSP-1, MSP-6, MSP-7, AMA1, PTRAMP and other proteins to prime merozoite for invasion	Subtilisin-like serine protease
Exoneme proteins					
SUB1	PF3D7_0507500	N	Exonemes/PV	Protease that processes MSP-1, MSP-6, MSP-7, AMA1, RAP1, MSRP2 and SERAs to prime merozoite for invasion	Subtilisin-like serine protease
Rhoptry neck proteins					
PfRh1	PF3D7_0402300	Y[a]	Rhoptry neck/surface	Binds to red cells via receptor Y	PfRh family
PfRh2a	PF3D7_1335400	Y	Rhoptry neck/surface	Binds to red cells via receptor Z	PfRh family
PfRh2b	PF3D7_1335300	Y	Rhoptry neck/surface	Binds to red cells via receptor Z	PfRh family
PfRh4	PF3D7_0424200	Y	Rhoptry neck/surface	Binds to red cells via complement receptor 1	PfRh family
PfRh5	PF3D7_0424100	N	Rhoptry neck/surface forms complex with Ripr	Binds to red cells via Basigin	Classed as PfRh family but lacks homology and no transmembrane so likely functionally distinct
RON2	PF3D7_1452000	ND	Rhoptry neck/into red cell membrane	Inserted in red cell membrane at invasion, forms complex at tight junction with RON proteins and AMA-1	Multipass transmembrane protein
RON3	PF3D7_1252100	ND	Rhoptry neck/into red cell	Likely also forms complex at tight junction with other RON proteins and AMA-1	No data
RON4	PF3D7_1116000	ND	Rhoptry neck/into red cell	Injected into red cell, binds to RON2 and forms a complex at tight junction with RON proteins and AMA-1	Binds to AMA1 via hydrophobic groove
RON5	PF3D7_0817700	ND	Rhoptry neck/into red cell	Forms complex at tight junction with RON proteins and AMA-1	No data
ASP	PF3D7_0405900	ND	Rhoptry neck/surface	Not known; has putative GPI anchor	Sushi domains

N, knockout attempt unsuccessful; Y, knockout generated; ND, knockout not attempted; PV, parasitophorous vacuole; MSP, merozoite surface protein
[a]EBL and PfRh families show overlap in function and, while individually nonessential, overall are essential.

invasion (Sanders et al., 2005). Several include domains suggesting that they are involved in protein–protein interactions. This includes Duffy binding–like (DBL) or erythrocyte binding–like (EBL) domains that are specific to *Plasmodium* spp. and present in many proteins of diverse function from invasion to postinvasion remodeling (Haynes et al., 1988; Adams et al., 1992) and cytoadherence (Su et al., 1997). Others include EGF (Savage et al., 1972) and six-cysteine (6-Cys) domains again implicated in protein–protein interactions (Ishino et al., 2005). The 6-Cys family is related to the surface antigen (SAG)-related sequence (SRS) superfamily found in coccidian members of the apicomplexan phylum (Gerloff et al., 2005; Arredondo et al., 2012).

Since the identification of the first merozoite surface protein 1 (MSP-1; Holder, 1988), a greatly expanded repertoire of surface proteins has been assembled (Table 1 and Fig. 2 A; Cowman and Crabb, 2006). MSP-1 is the most abundant and functionally conserved protein on the merozoite and is associated with the parasite membrane via a GPI anchor (Gerold et al., 1996). Eight other surface-bound GPI-anchored proteins have been identified, some of which have EGF or 6-Cys domains (Table 1; Sanders et al., 2005). One of these is MSP-2, which lacks identifiable domains and is intrinsically unstructured, containing significant amounts of sequence polymorphism and amino acid repeats (Low et al., 2007).

Surface proteins that are indirectly associated with the merozoite surface can be divided into three groups that include MSP-3, MSP-7, and the serine repeat antigen (SERA) protease-like family (for review see Cowman and Crabb, 2006). The MSP-3 family consists of a group of proteins encoded by clustered genes, some of which share similar motifs and a leucine-rich zipper-like domain (Gardner et al., 2002; Pearce et al., 2005). MSP-3, MSP-6, and MSP-7 associate with the merozoite surface via binding to the major surface protein MSP-1 (Kauth et al., 2003, 2006). MSPDBL-1 and -2 are also related to MSP-3; however, they contain an additional EBL domain (Wickramarachchi et al., 2009; Hodder et al., 2012; Sakamoto et al., 2012). The MSP-7 family consists of MSP-7, which binds tightly to MSP-1 (Kauth et al., 2006), and there are also six related genes that could encode MSP-7–like proteins called MSRPs, one of which is expressed on the merozoite surface (MSRP2; Kadekoppala et al., 2010). The SERA proteins (of which there are nine members in *P. falciparum*) contain a papain-like protease domain but also have additional regions that are likely involved in protein–protein interactions with other GPI-anchored proteins such as MSP-1 (Aoki et al., 2002; Hodder et al., 2003).

Despite this abundance of proteins on the surface, their functions are not fully known, although it is clear that some are required for the survival of the parasite, as the corresponding gene cannot be disrupted and specific antibodies can directly inhibit invasion (Blackman et al., 1994; O'Donnell et al., 2000). MSP-1, itself essential (O'Donnell et al., 2000), shows some evidence for binding directly to the erythrocyte surface Band 3 (Goel et al., 2003); however, definitive proof of the mechanistic importance of this interaction is lacking. Increasing evidence suggests that proteins such as MSP-7 and -6 bind to MSP-1 as a multiprotein complex, facilitating the display of individual epitopes to the external environment (Kauth et al., 2003, 2006). Of note, MSP-1 undergoes a complex series of highly regulated proteolytic cleavages by subtilisin 1 and 2 to form its macromolecular complex (Koussis et al., 2009), with processing required for binding of proteins such as MSP-6 (Kauth et al., 2006). MSP-2 is also essential and has a strong tendency to self-associate to form fibrils, which suggests that it is responsible for the dense surface coat present on the merozoite seen by electron microscopy (Low et al., 2007). MSPDBL1 and -2 adhere specifically to the erythrocyte through their EBL domains and are consequently likely to be involved in initial merozoite interaction with the red cell surface (Wickramarachchi et al., 2009; Hodder et al., 2012; Sakamoto et al., 2012). Less clear are the SERA proteases. Though they share a papain-like protease domain, not all are predicted to have a functional active site (Hodder et al., 2003). Only SERA5 and -6 have proven refractory to genetic disruption (McCoubrie et al., 2007), highlighting SERA6, which retains the functional cysteine residue in the active site, as a probable protease that may play an important role in invasion.

An intriguing question is why the parasite invests so heavily in exposed macromolecular and antigenically diverse surface proteins. It is likely that some modulate host responses to assist in merozoite survival after release from the infected erythrocyte (Oeuvray et al., 1994), such as via release of an immunological "smoke screen" or blocking activity of the complement pathway. For example, a nonuniform geographical distribution of Knops blood group complement receptor 1 may be suggestive of selective pressures exerted by malaria to avoid complement-mediated detection (Moulds, 2002). Although there is no molecular evidence to support this (Tetteh-Quarcoo et al., 2012), it is likely that *Plasmodium* spp. have developed mechanisms to protect the merozoite against complement and other innate host responses, with extrinsic proteins being prime candidates for this function.

Molecules functioning directly in invasion

The dramatic and rapid process of committed red cell binding, reorientation to the parasite apical pole, and active invasion involve multiple *P. falciparum* proteins. These processes appear finely coordinated and dependent on step-wise release and processing of proteins that, unlike their surface counterparts, are released just prior to or contiguous with invasion (Singh et al., 2010; Riglar et al., 2011). The different subcellular localizations of each protein and subcompartmentalization within secretory organelles (rhoptries in particular; Richard et al., 2009) likely play a critical coordinating role. Indeed, segregation of proteins allows each to be stored and released onto the invading parasite surface "just in time" to generate functional invasion complexes (Alexander et al., 2006; Besteiro et al., 2009; Chen et al., 2011). This process is shared among several merozoite invasion proteins and may function so that essential complexes are exposed to potential immune detection for a minimum amount of time.

The proteins that govern merozoite invasion can be loosely divided into two classes: adhesins that function as ligands binding

directly to specific receptors on the erythrocyte and invasins that function in the invasive process but do not necessarily bind directly to receptors on the host cell (Fig. 2 B and Table 1). Adhesins are located in both micronemes and rhoptries, and are in general *Plasmodium*-specific or provide cell specificity–restricting parasites (in the case of merozoite invasion) to the erythroid lineage (for reviews see Cowman and Crabb, 2006; Tham et al., 2012). Currently the main adhesins identified belong to two protein families that include the EBL and reticulocyte binding–like homologues (PfRh), localizing to the micronemes and neck of the rhoptries, respectively (Sim et al., 1990; Orlandi et al., 1992; Rayner et al., 2000; Triglia et al., 2001; Duraisingh et al., 2003). Different members of these adhesins bind to specific receptors, with EBA-175, Ebl1, and EBA-140 (also known as Baebl) binding to glycophorin A, B, and C, respectively (Sim et al., 1994; Lobo et al., 2003; Maier et al., 2003; Mayer et al., 2009). PfRh4 binds to complement receptor 1 (Tham et al., 2010). The PfRh and EBL protein families play an important role in phenotypic variation that allows different strains of *P. falciparum* to invade using alternative host receptors (Sim et al., 1990; Orlandi et al., 1992; Rayner et al., 2000; Triglia et al., 2001; Duraisingh et al., 2003).

The protein PfRh5 has recently been defined as an adhesin that binds erythrocyte surface CD147 or basigin (Crosnier et al., 2011). It is classified as a member of the PfRh family; however, it has no transmembrane region (present in all other PfRh family members), is broadly refractory to disruption, and shows little homology, suggesting that it may be functionally distinct (Hayton et al., 2008; Baum et al., 2009). Indeed, unlike other PfRhs, recent data has identified a conserved binding partner for PfRh5, the Rh5-interacting protein (PfRipr), which is localized in the micronemes and forms a complex with the rhoptry neck protein (Chen et al., 2011). Micronemal proteins from the thrombospondin-related anonymous protein (TRAP) family, including merozoite TRAP (MTRAP) and *Plasmodium* thrombospondin-related apical merozoite protein (PTRAMP), may provide a functional link to the internal parasite actin-myosin motor, bridging a gap between adhesins and invasins (Thompson et al., 2004; Baum et al., 2006; Uchime et al., 2012).

All invasins identified to date appear to be essential for merozoite invasion. Apical membrane antigen-1 (AMA1) is the best known of these proteins and is considered to be an important vaccine candidate that has progressed to clinical trials (Thera et al., 2011). As a micronemal protein (Narum and Thomas, 1994), AMA1 shares the same subcellular localization as the EBL family, although they are not present in the same individual organelles, which suggests the existence of micronemal subpopulations (Healer et al., 2002). AMA1 interacts with a set of rhoptry neck proteins (the RON complex) that comes together at the tight junction during invasion (Alexander et al., 2005, 2006; Besteiro et al., 2009; Richard et al., 2010; Lamarque et al., 2011; Tyler and Boothroyd, 2011). This pairing of proteins from different compartments appears to be a common theme with invasins and other critical components of erythrocyte entry (Chen et al., 2011).

The stages of invasion

Important steps required for merozoite invasion begin before egress from the host cell (either hepatocytes or erythrocytes), which entails a process of "priming" proteins for a new round of entry (Fig. 3). An essential subtilisin-like protease called PfSUB1 is discharged from discrete apical organelles termed exonemes into the parasitophorous vacuolar space (Yeoh et al., 2007). PfSUB1 is responsible for proteolysis of the SERA proteins (Arastu-Kapur et al., 2008; Koussis et al., 2009; Silmon de Monerri et al., 2011). Along with a second subtilisin (PfSUB2), PfSUB1 also mediates primary proteolytic processing of merozoite surface protein 1 (Barale et al., 1999; Koussis et al., 2009; Child et al., 2010), as well as several other merozoite surface proteins (Koussis et al., 2009). Although many of these proteolytic cleavage events appear to be essential for invasion (Child et al., 2010), their exact function has yet to be established.

Once the merozoite is released from the infected erythrocyte, it is exposed to low potassium levels. This triggers calcium release that activates secretion of adhesins and invasins from micronemes onto the parasite surface (Treeck et al., 2009; Singh et al., 2010; Srinivasan et al., 2011). When the protease-primed and activated merozoite encounters an erythrocyte, low-affinity interactions occur with the erythrocyte membrane, most likely governed by members of the merozoite surface class of proteins (Dvorak et al., 1975; Hodder et al., 2012). Among the likely candidates are MSPDBL1 and -2 and the 6-Cys protein family (Ishino et al., 2005; Sanders et al., 2005; Wickramarachchi et al., 2009; Sakamoto et al., 2012). Initial interaction involves major movement of the merozoite and dramatic ruffling of the erythrocyte membrane (Gilson and Crabb, 2009). It is not known, however, if these are parasite-specific processes or whether the merozoite signals change in the cytoskeleton of the erythrocyte, which is then responding to merozoite interaction (Zuccala and Baum, 2011). Long-standing dogma has traditionally placed the role of the erythrocyte as being passive in invasion; however, the dramatic physical deformations seen and recent implications from hepatocyte invasion may suggest otherwise (Gonzalez et al., 2009).

After initial interaction, irreversible attachment to the erythrocyte occurs at the apical end of the merozoite, probably through attachment of EBL and PfRh proteins. These appear to mediate commitment to invasion and trigger subsequent events leading to entry (Singh et al., 2010; Riglar et al., 2011; Srinivasan et al., 2011). Further subcompartmentalization of the rhoptries (after initial PfRh protein release) facilitates the stepwise function of proteins, commencing with the RON complex. This is both released and inserted into the erythrocyte, with RON2 acting as an anchor in the erythrocyte membrane for RON complex assembly, and as a likely traction point on which the merozoite bears for entry (Besteiro et al., 2011). This allows AMA1, which is present on the merozoite surface after release from the micronemes at egress, to complex with RON2, thus forming a link between the erythrocyte and parasite (Riglar et al., 2011). Formation of the junction likely triggers the release of the rhoptry bulb, providing proteins and lipids required for the parasitophorous vacuole membrane and

parasitophorous vacuole to establish the space into which the merozoite can move as it invades (Riglar et al., 2011). These steps likely require additional signaling events. AMA1 in particular is phosphorylated by cAMP-regulated protein kinase A, and it appears to be involved in signaling rhoptry release (Treeck et al., 2006; Leykauf et al., 2010).

There is still uncertainty about the core functional components of the tight junction linking the erythrocyte membrane, parasite, and internal actomyosin motor (Giovannini et al., 2011). AMA1 forms a ring that follows the tight junction together with the RON complex and has thus far proven refractory to genetic disruption in *P. falciparum* (Triglia et al., 2000; Riglar et al., 2011). In *Plasmodium berghei*, a malaria species that infects rodents, AMA1 also appears to be essential for merozoite invasion of mouse erythrocytes; however, remarkably it is not essential for sporozoite invasion of hepatocytes in contrast to RON4. Similarly, invasion of fibroblasts by *Toxoplasma gondii* can still occur in the absence of AMA1 function (Giovannini et al., 2011). This suggests that while AMA1 may link the host cell and invading parasite through its interaction with RON2, it may not play an essential role in tight junction architecture, for sporozoite invasion of hepatocytes and also by inference for merozoite invasion of erythrocytes. Linkage between the junction and internal parasite motor that drives invasion (Baum et al., 2006) does not appear to be direct, as demonstrated by displacement of engaged actin at the junction (Angrisano et al., 2012), which suggests that other proteins involved in the structure and function of the tight junction remain to be discovered.

The force that drives invasion is produced by a single-headed myosin attached to the double membrane IMC via a complex of proteins forming a substrate against which it can be braced (Opitz and Soldati, 2002; Baum et al., 2006; Bullen et al., 2009). One of these proteins, GAP45, spans the space between the IMC and the plasma membrane, to which it is attached by myristyl and palmitoyl moieties (Frénal et al., 2010). Actin filaments also concentrate at this site, forming a ring-like distribution at the tight junction of the invading merozoite trailing the RON complex (Angrisano et al., 2012). This provides a substrate with which the myosin head can interact to generate force for movement, propelling the merozoite into the space generated by release of the rhoptries and development of the parasitophorous vacuole membrane. The tight junction is then pulled across the surface of the merozoite, drawing with it the erythrocyte membrane until the parasite cell is sealed, presumably by fusion of the parasitophorous vacuole and erythrocyte membranes (Riglar et al., 2011).

Conclusions

The identity of many of the proteins involved in merozoite invasion are now known together with some understanding of when they are required; however, there is still much to understand with respect to their functions, the sequence in which they act, and how processes link to produce the coherent and remarkably rapid process of invasion. Technical improvements in genetic technologies as well as live and super-resolution microscopy have expanded our armamentarium for dissecting this important infectious agent (Sanders et al., 2005; Riglar et al., 2011). Despite this progress in understanding, there are gaps to be filled in our knowledge. The interaction of the merozoite with erythrocytes is dynamic, with parasite and host cell undergoing dramatic changes (Gilson and Crabb, 2009). The identity of the parasite ligands and host receptors involved in this process are unknown, although there are potential culprits. Commitment to invasion by a merozoite occurs once the apical end interacts with the erythrocyte, and although EBL and PfRh proteins appear to be involved in this signaling, there are gaps in our understanding. Once the merozoite has activated invasion, it inserts the RON complex and potentially other proteins under and into the erythrocyte membrane. Current evidence would suggest that a hole in the erythrocyte membrane is not generated for injection of proteins (of note, no perforin-like membrane attack proteins are expressed in this lifecycle stage; Kaiser et al., 2004), and therefore may occur via some form of membrane fusion. The tight junction necessarily must link the host cell and parasite membrane to the actomyosin motor of the merozoite, with the only protein so far suggested to be involved in this linkage being MTRAP. The RON complex and AMA1 also appear to play key roles at the junction, though the role of AMA1 as a link between erythrocyte and the parasite surface is now a matter for debate; however, there is no evidence suggesting that these bind to the actomyosin motor either directly or indirectly (Angrisano et al., 2012). It is therefore likely that other proteins must be involved in the formation and structure of the tight junction. Finally, as the merozoite moves into the red cell, the erythrocyte membrane and the newly formed parasitophorous vacuole membrane must fuse to seal the invasion process. There is no information on how this membrane fusion process is initiated and controlled, and although it may involve dynamin-like proteins, none have been identified.

The case for a blood stage vaccine, and global need, is still profound. An increased understanding of merozoite biology and the intricacies involved in the exquisite process of invasion will certainly provide critical knowledge for future development of novel and synergistic strategies to target erythrocyte entry as a vehicle for treating and controlling malaria.

We apologize to many researchers in this field whose work we have not been able to cite directly because of the limits of space.

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