

## Inside Scoop on Outside Proteins

Jeffrey D. Dvorin

Division of Infectious Diseases, Boston Children's Hospital, Boston, Massachusetts, USA, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA

**Invasion into red blood cells is an essential step in the life cycle of parasites that cause human malaria. Antibodies targeting the key parasite proteins in this process are important for developing a protective immune response. In the current issue, Boyle and colleagues provide a detailed examination of *Plasmodium falciparum* invasion and specifically illuminate the fate of surface-exposed parasite proteins during and immediately after invasion.**

Human malaria remains a leading cause of death and disease worldwide. Recent estimates suggest that there were 225 million cases of malaria in 2009, with more than 1 million deaths in 2010 (1, 2). The parasite *Plasmodium falciparum* causes the most severe forms of malaria. Resistance to existing antimalarial medications is a constant and continually emerging hurdle to the effective treatment of malaria. At the same time, efforts to find a broadly effective vaccine providing sustained protection have not yet been successful. For these reasons, and many more, a more intricate understanding of the molecular details of the parasite life cycle is needed. Knowledge of the molecular mechanism of parasite invasion, replication, and egress will hopefully provide new targets for antimalarial therapeutics and vaccines. The work described in this issue by Boyle and colleagues (3) provides a new layer of complexity in our understanding of *P. falciparum* invasion into human red blood cells (RBCs).

### PLASMODIUM FALCIPARUM: AN EVASIVE INVADER

After a susceptible person is bitten by an infected female *Anopheles* mosquito, *P. falciparum* sporozoites travel to the liver and invade hepatic cells. Inside the hepatocyte, the sporozoite differentiates and produces thousands of RBC-invasive merozoites (4). Once released from the protected intracellular environment, a merozoite has a very limited window of time to find and invade a RBC or risks clearance by the host reticuloendothelial system and/or inactivation by antibodies. Several elegant studies have described the basic steps of merozoite invasion of RBCs (5–12) (Fig. 1). The predominant model of parasite invasion proposes that the merozoite reversibly binds the surface of the RBC via multiple low-affinity interactions between parasite-expressed merozoite surface proteins (MSPs) and RBC surface proteins (e.g., Band 3) or heparin-like glycosaminoglycans (13–15). The invading merozoite must reorient itself to bring the apical end, with its associated apical organelles (rhoptries, micronemes, and dense granules), into direct contact with the RBC surface. Reorientation likely requires the action of *P. falciparum* apical membrane protein 1 (PfAMA1) (7). Interestingly, the “receptor” for PfAMA1 is another parasite protein, RON2, that is injected into the host side of the RBC membrane (16, 17). Following reorientation, the merozoite forms an irreversible attachment to the RBC, known as the tight junction, which is made in part from the PfAMA1-RON2 interaction. The formation of the tight junction likely involves multiple interactions between parasite ligands located in the micronemes and rhoptries and RBC surface receptors (5, 6, 9, 18–21). The exact sequence of events surrounding the engagement of

apical organelle receptor proteins, the timing of the release of organelle contents, and the trigger for their release remain incompletely resolved (22–24). The final step of invasion is junction movement and formation of the intracellular parasitophorous vacuole. This stage of invasion involves actinomyosin motor complexes inside the parasite (12). The signal to initiate actinomyosin-dependent movement after junction formation remains unknown but likely involves calcium signaling.

### PLEASE REMOVE YOUR COAT AT THE DOOR

Because merozoites actively power themselves into the host cell instead of relying on the host endocytic machinery, the multiple parasite ligand-host cell receptor interactions must be disengaged to allow the parasite to “drive” into the RBC, form its parasitophorous vacuole, and reseal the host cell plasma membrane. It has been hypothesized that in multiple Apicomplexa, including *P. falciparum*, *Toxoplasma gondii*, *Neospora caninum*, *Eimeria tenella*, and *Cryptosporidium parvum*, parasite-derived proteases catalyze the removal of the invading zoite surface proteins during invasion (25). Prior to their identification, the hypothesized proteases that cleaved surface proteins at the tight junction and at the posterior end of the invading zoite were called “sheddases” and “cap proteases.” The major sheddase has been identified as a subtilisin-like serine protease, PfSUB2 (26). More recently, the cap protease was identified as a rhomboid-type protease, PfROM4 (27). The rhomboid proteases are intramembrane serine proteases that cleave their substrates within the transmembrane domain. Another essential rhomboid protease, PfROM1, that cleaves multiple invasion ligands has been identified in *P. falciparum* (28, 29); however, it is unclear if this protease is required for priming of invasion ligands prior to invasion or for surface shedding after receptor engagement.

Some of the remaining “stubs” of merozoite surface proteins are known to be maintained by the parasite for several hours. The glycosylphosphatidylinositol (GPI)-anchored stub of MSP1,

Published ahead of print 16 December 2013

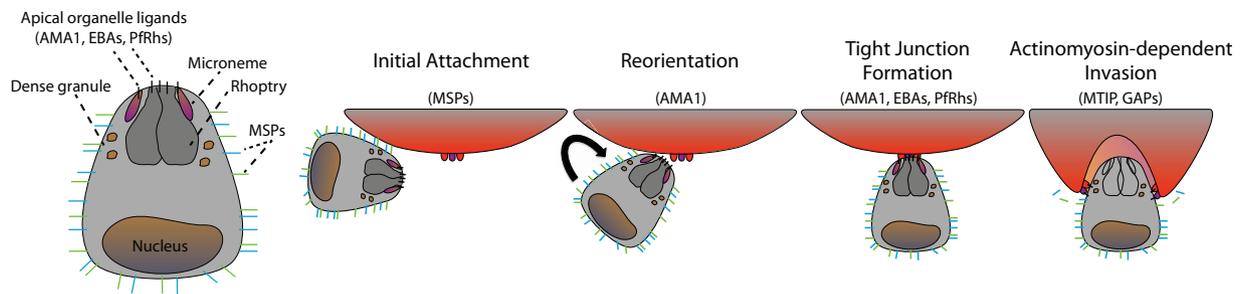
Editor: J. H. Adams

Address correspondence to Jeffrey D. Dvorin, jeffrey.dvorin@childrens.harvard.edu.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.01542-13

The views expressed in this Commentary do not necessarily reflect the views of the journal or of ASM.



**FIG 1** Schematic of *P. falciparum* invasion. The merozoite initially attaches to the RBC surface via MSPs. The parasite AMA1 protein is important for reorientation. An irreversible tight junction forms between parasite-parasite protein interactions (AMA1/RON2) and parasite-host RBC interactions. The important parasite proteins include the erythrocyte binding antigen (EBA) proteins (e.g., EBA175) and the reticulocyte binding protein homolog (Pfrh) proteins (e.g., Pfrh2b). Following tight junction formation, the parasite actinomyosin motor actively invades the RBC. The parasite sheddase is likely located at the tight junction, releasing the surface proteins during invasion. MTIP, myosin A tail domain interacting protein; GAPs, glideosome-associated proteins.

MSP<sub>1-19</sub>, is known to be retained by the parasite for several hours after invasion and may play a role in the formation of the food vacuole (30, 31). A role for the stub of AMA1 in *T. gondii* has been similarly hypothesized (32). Aside from the stub functions described above, the current paradigm for parasite invasion is that most (or all) of the surface proteins are removed by parasite proteases during entry. Boyle and colleagues challenge this paradigm in this issue, demonstrating that at least two of the merozoite surface proteins, MSP2 and MSP4, are retained intact on the surface of the merozoite during and immediately after invasion (3). In a previous study from this same group, they described a method to purify viable and invasive extracellular (“free”) merozoites (33). Shortly after this initial description, the free-merozoite technique was used to visually evaluate the steps of parasite invasion by using superresolution microscopy (22). In their current study, Boyle and colleagues utilize these invasive merozoites to analyze the fate of surface membrane proteins and membrane-associated proteins immediately prior to, during, and after invasion. It is important to note that the use of free merozoites followed by rapid fixation allows analysis of events that occur within seconds to minutes after initial contact between the parasite and the RBC. By generating a series of both monoclonal and polyclonal antisera against MSP2 and MSP4, Boyle and colleagues demonstrate that both GPI-anchored surface proteins are kept intact on the interior and exterior sides of the tight junction, thus escaping the action of the sheddases. While antibodies directed against MSP2 did not inhibit parasite invasion or growth, bound antibodies were carried inside the parasitophorous vacuole. MSP2 was rapidly degraded following the completion of invasion (with or without bound antibodies and by an unknown mechanism), but the anti-MSP2 antibodies were retained for several additional hours without an ill effect on parasite growth. Polyclonal antibodies against MSP4 caused a modest decrease in parasite invasion. In contrast to the rapid, postinvasion degradation of MSP2, MSP4 is retained, likely fully intact, by the parasite for up to 19 h. Using a series of immunofluorescence and immunoblot analysis experiments, Boyle and colleagues convincingly demonstrate that MSP4 is maintained for at least 19 h and is then degraded in the following 5 h. This suggests the interesting possibility that MSP4 has a specific role in the first 20 h postinvasion, a period of time when the parasite must remodel the host RBC to allow specific trafficking of parasite proteins to the RBC surface.

### LIFE BEYOND THE SHEDDASE?

The reasons for the lack of shedding of MSP2 and MSP4 during invasion remain unknown. While it is possible that these proteins have no postinvasion function, it seems more likely that the essential MSP2 and MSP4 proteins, and potentially other surface proteins, have essential postinvasion functions. Boyle and colleagues bring this intriguing hypothesis to the forefront in this issue. This provides a new fundamental biological step in the parasite life cycle that can be investigated and hopefully targeted and/or blocked by new vaccines or antimalarial therapeutics. Armed with this detailed molecular knowledge of the parasite life cycle, the research community can design the next set of experiments and, more importantly, the next techniques to block parasite replication in humans.

### ACKNOWLEDGMENTS

J.D.D. is supported by grants from the NIH (DP2AI112219-01), the Charles H. Hood Foundation, and the Boston Children’s Hospital Faculty Development Program.

### REFERENCES

1. Cibulskis RE, Aregawi M, Williams R, Otten M, Dye C. 2011. Worldwide incidence of malaria in 2009: estimates, time trends, and a critique of methods. *PLoS Med.* 8:e1001142. <http://dx.doi.org/10.1371/journal.pmed.1001142>.
2. Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez AD. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet* 379:413–431. [http://dx.doi.org/10.1016/S0140-6736\(12\)60034-8](http://dx.doi.org/10.1016/S0140-6736(12)60034-8).
3. Boyle MJ, Langer C, Chan J, Hodder AN, Coppel RN, Anders RF, Beeson JG. 2014. Sequential processing of merozoite surface proteins during and after erythrocyte invasion by *Plasmodium falciparum*. *Infect. Immun.* 82:924–936. <http://dx.doi.org/10.1128/IAI.00866-13>.
4. Miller LH, Baruch DI, Marsh K, Doumbo OK. 2002. The pathogenic basis of malaria. *Nature* 415:673–679. <http://dx.doi.org/10.1038/415673a>.
5. Gaur D, Mayer DC, Miller LH. 2004. Parasite ligand-host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. *Int. J. Parasitol.* 34:1413–1429. <http://dx.doi.org/10.1016/j.ijpara.2004.10.010>.
6. Cowman A, Crabb B. 2006. Invasion of red blood cells by malaria parasites. *Cell* 124:755–766. <http://dx.doi.org/10.1016/j.cell.2006.02.006>.
7. Mitchell GH, Thomas AW, Margos G, Dluzewski AR, Bannister LH. 2004. Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *Infect. Immun.* 72:154–158. <http://dx.doi.org/10.1128/IAI.72.1.154-158.2004>.
8. Rayner JC, Huber CS, Galinski MR, Barnwell JW. 2004. Rapid evolution of an erythrocyte invasion gene family: the *Plasmodium reichenowi* re-

- ticulocyte binding like (RBL) genes. *Mol. Biochem. Parasitol.* 133:287–296. <http://dx.doi.org/10.1016/j.molbiopara.2003.10.017>.
9. Sim BK, Chitnis CE, Wasniowska K, Hadley TJ, Miller LH. 1994. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. *Science* 264:1941–1944. <http://dx.doi.org/10.1126/science.8009226>.
  10. Rayner JC, Galinski MR, Ingravallo P, Barnwell JW. 2000. Two *Plasmodium falciparum* genes express merozoite proteins that are related to *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins involved in host cell selection and invasion. *Proc. Natl. Acad. Sci. U. S. A.* 97:9648–9653. <http://dx.doi.org/10.1073/pnas.160469097>.
  11. Adams JH, Sim BK, Dolan SA, Fang X, Kaslow DC, Miller LH. 1992. A family of erythrocyte binding proteins of malaria parasites. *Proc. Natl. Acad. Sci. U. S. A.* 89:7085–7089. <http://dx.doi.org/10.1073/pnas.89.15.7085>.
  12. Baum J, Richard D, Healer J, Rug M, Krnajski Z, Gilberger T-W, Green JL, Holder AA, Cowman AF. 2006. A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *J. Biol. Chem.* 281:5197–5208. <http://dx.doi.org/10.1074/jbc.M509807200>.
  13. Boyle MJ, Richards JS, Gilson PR, Chai W, Beeson JG. 2010. Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood* 115:4559–4568. <http://dx.doi.org/10.1182/blood-2009-09-243725>.
  14. Goel VK, Li X, Chen H, Liu SC, Chishti AH, Oh SS. 2003. Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* 100:5164–5169. <http://dx.doi.org/10.1073/pnas.0834959100>.
  15. Li X, Chen H, Oo TH, Daly TM, Bergman LW, Liu S-C, Chishti AH, Oh SS. 2004. A co-ligand complex anchors *Plasmodium falciparum* merozoites to the erythrocyte invasion receptor band 3. *J. Biol. Chem.* 279:5765–5771. <http://dx.doi.org/10.1074/jbc.M308716200>.
  16. Richard D, Macrauld CA, Riglar DT, Chan J-A, Foley M, Baum J, Ralph SA, Norton RS, Cowman AF. 2010. Interaction between *Plasmodium falciparum* invasion membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. *J. Biol. Chem.* 285:14815–14822. <http://dx.doi.org/10.1074/jbc.M109.080770>.
  17. Srinivasan P, Beatty WL, Diouf A, Herrera R, Ambroggio X, Moch JK, Tyler JS, Narum DL, Pierce SK, Boothroyd JC, Haynes JD, Miller LH. 2011. Binding of *Plasmodium* merozoite proteins RON2 and AMA1 triggers commitment to invasion. *Proc. Natl. Acad. Sci. U. S. A.* 108:13275–13280. <http://dx.doi.org/10.1073/pnas.1110303108>.
  18. Okoyeh J, Pillai CR, Chitnis CE. 1999. *Plasmodium falciparum* field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A. *Infect. Immun.* 67:5784–5791.
  19. Dolan SA, Proctor JL, Okubo DW, Wellem TE, Miller LH. 1994. Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes. *Mol. Biochem. Parasitol.* 64:55–63. [http://dx.doi.org/10.1016/0166-6851\(94\)90134-1](http://dx.doi.org/10.1016/0166-6851(94)90134-1).
  20. Dvorin JD, Bei AK, Coleman BI, Duraisingh MT. 2010. Functional diversification between two related *Plasmodium falciparum* merozoite invasion ligands is determined by changes in the cytoplasmic domain. *Mol. Microbiol.* 75:990–1006. <http://dx.doi.org/10.1111/j.1365-2958.2009.07040.x>.
  21. Gilberger T-W, Thompson JK, Triglia T, Good RT, Duraisingh MT, Cowman AF. 2003. A novel erythrocyte binding antigen-175 paralogue from *Plasmodium falciparum* defines a new trypsin-resistant receptor on human erythrocytes. *J. Biol. Chem.* 278:14480–14486. <http://dx.doi.org/10.1074/jbc.M211446200>.
  22. Riglar DT, Richard D, Wilson DW, Boyle MJ, Dekiwadia C, Turnbull L, Angrisano F, Marapana DS, Rogers KL, Whitchurch CB, Beeson JG, Cowman AF, Ralph SA, Baum J. 2011. Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. *Cell Host Microbe* 9:9–20. <http://dx.doi.org/10.1016/j.chom.2010.12.003>.
  23. Singh S, Alam MM, Pal-Bhowmick I, Brzostowski JA, Chitnis CE. 2010. Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog.* 6:e1000746. <http://dx.doi.org/10.1371/journal.ppat.1000746>.
  24. Gao X, Gunalan K, Yap SS, Preiser PR. 2013. Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*. *Nat. Commun.* 4:2862. <http://dx.doi.org/10.1038/ncomms3862>.
  25. Carruthers VB, Blackman MJ. 2005. A new release on life: emerging concepts in proteolysis and parasite invasion. *Mol. Microbiol.* 55:1617–1630. <http://dx.doi.org/10.1111/j.1365-2958.2005.04483.x>.
  26. Harris PK, Yeoh S, Dluzewski AR, O'Donnell RA, Withers-Martinez C, Hackett F, Bannister LH, Mitchell GH, Blackman MJ. 2006. Molecular identification of a malaria merozoite surface sheddase. *PLoS Pathog.* 1:241–251. <http://dx.doi.org/10.1371/journal.ppat.0010029>.
  27. O'Donnell RA, Hackett F, Howell SA, Treeck M, Struck N, Krnajski Z, Withers-Martinez C, Gilberger TW, Blackman MJ. 2006. Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. *J. Cell Biol.* 174:1023–1033. <http://dx.doi.org/10.1083/jcb.200604136>.
  28. Baker RP, Wijetilaka R, Urban S. 2006. Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathog.* 2:e113. <http://dx.doi.org/10.1371/journal.ppat.0020113>.
  29. Singh S, Plasmeyer M, Gaur D, Miller LH. 2007. Mononeme: a new secretory organelle in *Plasmodium falciparum* merozoites identified by localization of rhomboid-1 protease. *Proc. Natl. Acad. Sci. U. S. A.* 104:20043–20048. <http://dx.doi.org/10.1073/pnas.0709999104>.
  30. Blackman MJ, Holder AA. 1992. Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. *Mol. Biochem. Parasitol.* 50:307–315. [http://dx.doi.org/10.1016/0166-6851\(92\)90228-C](http://dx.doi.org/10.1016/0166-6851(92)90228-C).
  31. Dluzewski AR, Ling IT, Hopkins JM, Grainger M, Margos G, Mitchell GH, Holder AA, Bannister LH. 2008. Formation of the food vacuole in *Plasmodium falciparum*: a potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1<sub>19</sub>). *PLoS One* 3:e3085. <http://dx.doi.org/10.1371/journal.pone.0003085>.
  32. Santos JM, Ferguson DJ, Blackman MJ, Soldati-Favre D. 2011. Intramembrane cleavage of AMA1 triggers *Toxoplasma* to switch from an invasive to a replicative mode. *Science* 331:473–477. <http://dx.doi.org/10.1126/science.1199284>.
  33. Boyle MJ, Wilson DW, Richards JS, Riglar DT, Tetteh KK, Conway DJ, Ralph SA, Baum J, Beeson JG. 2010. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. *Proc. Natl. Acad. Sci. U. S. A.* 107:14378–14383. <http://dx.doi.org/10.1073/pnas.1009198107>.