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Molecular Signaling Involved in Entry and Exit of Malaria Parasites from Host Erythrocytes

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During the blood stage, *Plasmodium* spp. merozoites invade host red blood cells (RBCs), multiply, exit, and reinvade uninfected RBCs in a continuing cycle that is responsible for all the clinical symptoms associated with malaria. Entry into (invasion) and exit from (egress) RBCs are highly regulated processes that are mediated by an array of parasite proteins with specific functional roles. Many of these parasite proteins are stored in specialized apical secretory vesicles, and their timely release is critical for successful invasion and egress. For example, the discharge of parasite protein ligands to the apical surface of merozoites is required for interaction with host receptors to mediate invasion, and the timely discharge of proteases and pore-forming proteins helps in permeabilization and dismantling of limiting membranes during egress. This review focuses on our understanding of the signaling mechanisms that regulate apical organelle secretion during host cell invasion and egress by malaria parasites. The review also explores how understanding key signaling mechanisms in the parasite can open opportunities to develop novel strategies to target *Plasmodium* parasites and eliminate malaria.

Plasmodium falciparum has a complex life cycle involving multiple stages in two hosts, namely, the vertebrate human host and the invertebrate *Anopheles* mosquito, which also serves as the vector for transmission. *P. falciparum* sporozoites are introduced into the human host following a bite by an infected female *Anopheles* mosquito in search of a blood meal. The injected sporozoites traverse through the bloodstream to the liver where they infect hepatocytes, multiply, and differentiate into merozoites. The merozoites are released into the bloodstream in membrane-bound packets called merosomes, which protect the parasite from host immune mechanisms. Merozoites emerge from merosomes in the bloodstream and go on to invade host red blood cells (RBCs) within which they develop and multiply by schizogeny over a period of 36–48 hours. Once the next generation of merozoites has developed, the mature schizont ruptures and merozoites egress in a highly synchronized manner. Merozoite egress requires disruption of the RBC cytoskeleton and rupture of multi-

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ple limiting membranes (Salmon et al. 2001; Wickham et al. 2003). Key parasite effectors that mediate egress include proteases as well as perforin-like proteins (Blackman 2008; Roiko and Carruthers 2009, 2013; Agarwal et al. 2012; Garg et al. 2013). Many of these effectors are localized in apical organelles and are secreted in a timely, regulated manner to initiate egress of merozoites from mature schizonts. The released merozoites go on to invade uninfected erythrocytes to continue blood stage multiplication.

Invasion of host erythrocytes by *P. falciparum* merozoites is also a complex multistep process that involves initial attachment, apical reorientation, junction formation, development of a nascent vacuole, and movement of the parasite into the vacuole, followed by resealing of the vacuole so that the parasite is surrounded by a vacuolar membrane at the end of invasion (Dvorak et al. 1975; Aikawa et al. 1978; Cowman and Crabb 2006; Gilson and Crabb 2009; Riglar et al. 2011; Baum 2013). Many of the parasite ligands that bind host receptors to mediate these steps during invasion are initially localized in the micronemes and rhoptries at the apical end of merozoites. They are secreted to the surface of the merozoite in a highly regulated manner to enable receptor binding and invasion (Singh et al. 2010; Gaur and Chitnis 2011; Riglar et al. 2011; Singh and Chitnis 2012; Sharma and Chitnis 2013).

Here, we will review our current understanding of the signals and signaling mechanisms that regulate the highly coordinated processes of parasite egress and host cell invasion. This review will focus on our understanding of the key signaling mechanisms that trigger the release of parasite proteins from apical organelles to mediate parasite egress and invasion. We will include information from both malaria parasites as well as the related apicomplexan parasite, *Toxoplasma gondii*, which shares many common features in the process of host cell invasion. Finally, we will discuss how a better understanding of signaling processes during invasion and egress may provide novel targets for intervention to interrupt the malaria parasite life cycle and eliminate malaria.

MOLECULAR PLAYERS AND SIGNALS FOR EGRESS OF *Plasmodium falciparum* MEROZOITES FROM MATURE SCHIZONTS

Egress of *P. falciparum* merozoites from RBCs requires breaching of multiple barriers including the parasitophorous vacuole membrane (PVM), the host cytoskeleton, and the host plasma membrane (HPM). Parasite egress is synchronized with completion of its replicative cycle when next generation of invasive parasites have developed fully. Several parasite proteases have been identified as key effectors of the egress process in apicomplexan blood-stage parasites (Blackman 2008; Roiko and Carruthers 2009). In addition, perforin-like proteins with homology with mammalian perforins are involved in egress by *T. gondii* tachyzoites (TgPLP) as well as *P. falciparum* merozoites (PfPLP) (Kafsack et al. 2009, 2010; Garg et al. 2013). The PLPs and the subtilisin-like protease, PfSub1, which mediate egress, are located in micronemes and exonemes, respectively (Yeoh et al. 2007; Agarwal et al. 2012; Garg et al. 2013). What are the signals and signaling mechanisms that trigger release of these effectors to initiate egress?

The phytohormone abscisic acid (ABA) was identified as the internal signal that triggers egress of *T. gondii* tachyzoites (Nagamune et al. 2008). In plants, ABA acts as a hormone that mediates growth and responses to environmental cues through cyclic ADP-ribose (cADPR) and calcium (Ca^{2+}) fluxes (Wu et al. 1997). In *T. gondii*, ABA induces the production of cADPR, which activates Ca^{2+} release from an internal membrane-bound pool (probably the endoplasmic reticulum [ER]) leading to secretion of microneme proteins such as TgPLP (Chini et al. 2005; Nagamune et al. 2008). Although the parasite genome has candidate genes for ABA synthesis, the presence of the entire biosynthetic pathway remains to be confirmed. Reflecting its plant heritage, ABA is most likely synthesized in the apicoplast, an organelle found in apicomplexans that is derived from an algal endosymbiont. Interestingly, ABA levels spike late in schizogeny just before parasite egress. Thus, the steep increase in production of ABA at the end of the replicative cycle might

serve as an intrinsic cue for egress. The herbicide fluridone, which inhibits ABA synthesis, blocks egress of *T. gondii* tachyzoites (Nagamune et al. 2008). Furthermore, mice treated with fluridone survive inoculation with a lethal dose of *Toxoplasma*. Fluridone also blocks Ca^{2+}-mediated egress of *P. falciparum* merozoites from mature schizonts (S Singh and CE Chitnis, unpubl.), suggesting a conserved role for ABA in regulating egress in apicomplexan parasites (Fig. 1).

Rupture of limiting membranes during egress exposes *T. gondii* tachyzoites to a drop in the concentration of K^+ ($[K^+]$), which activates tachyzoite motility leading to rapid exit of mature tachyzoites (Moudy et al. 2001). Precisely how the tachyzoite senses $[K^+]$ is not known, although phospholipase C (PLC), cytosolic $[Ca^{2+}]$ levels, and several Ca^{+2}-responsive proteins are thought to play a role (Moudy et al. 2001). The levels of $[K^+]$ in the environment may be a natural external cue for tachyzoite egress at the end of the replicative cycle. However, parasites have also been observed to exit host cells in the absence of parasite motility or low $[K^+]$ (Lavine and Arrizabalaga 2008; Abkarian et al. 2011). In case of malaria parasites, osmotic stress and host cell membrane tension may induce egress (Abkarian et al. 2011). Under normal circumstances, however, it is likely that the parasite uses intrinsic as well as extrinsic cues such as ABA and $[K^+]$, respectively, as signals to trigger escape.

Several recent studies have provided compelling evidence for a role for proteases in egress of *Plasmodium* spp. merozoites (Blackman 2004, 2008). Protease inhibitors have proved invaluable tools in analyzing the role of proteases in *Plasmodium* biology and have shown that egress is a protease-dependent function. Although multiple models for merozoite egress have been proposed, evidence is accumulating in support of a model in which the PVM is disrupted before the RBC membrane (RBCM) (Winograd et al. 1999; Wickham et al. 2003). Further evidence for a model in which the parasite egresses in a stepwise manner came from observations that disruption of each limiting membrane is sensitive to different protease inhibitors (Salmon et al. 2001; Wickham et al. 2003; Soni et al. 2005; Millholland et al. 2011). Whereas the cysteine and serine protease inhibitors, leupeptin and antipain, block RBCM disruption, the cysteine protease inhibitor E-64 blocks PVM disruption (Glushakova et al. 2008). The targets of these inhibitors, however, remain to be determined.

Other proteases involved in egress include DPAP3, a cathepsin-C like cysteine protease, which is required for maturation of PfSUB1, a subtilisin-like protease implicated in egress (Arastu-Kapur et al. 2008). PfSUB1 is an essential protein that is expressed in late-blood-stage parasites and is secreted into the PV from apical merozoite organelles called exonemes before egress (Yeoh et al. 2007). Once in the PV, PfSUB1 processes serine-rich antigen 5 (SERA5) and potentially other serine-repeat antigen (SERA) proteins (Yeoh et al. 2007; Arastu-Kapur et al. 2008). The SERAs are PV proteins with papain-like putative protease domains. Although evidence of proteolytic activity has yet to be shown by SERAs, it is possible that PfSub1 processing of SERA5 and other SERAs results in their activation and proteolysis of host cytoskeletal proteins to aid in parasite egress (Yeoh et al. 2007). Alternatively, SERAs might activate other parasite or host proteins that promote parasite egress. PfSERA5 processing from a precursor to mature form is linked to egress because this putative activation step occurs coincident with parasite exit from the erythrocyte (Blackman 2008). Inhibition of PfSERA5 by a cyclical peptide halts late-stage development of intraerythrocytic parasites possibly by blocking egress (Fairlie et al. 2008).

A host calcium-dependent protease, calpain-1, is also required for efficient parasite egress of *Plasmodium* spp. merozoites and *Toxoplasma* tachyzoites (Olaya and Wasserman 1991; Chandramohanadas et al. 2009). Treatment of *P. falciparum* infected erythrocytes with an irreversible cysteine protease inhibitor, DCG04, did not affect parasite maturation but prevented parasite release from the host cell (Chandramohanadas et al. 2009). Selective extraction of treated cells identified host calpain-1 as the target of the inhibitor. Calpain-1 was observed in

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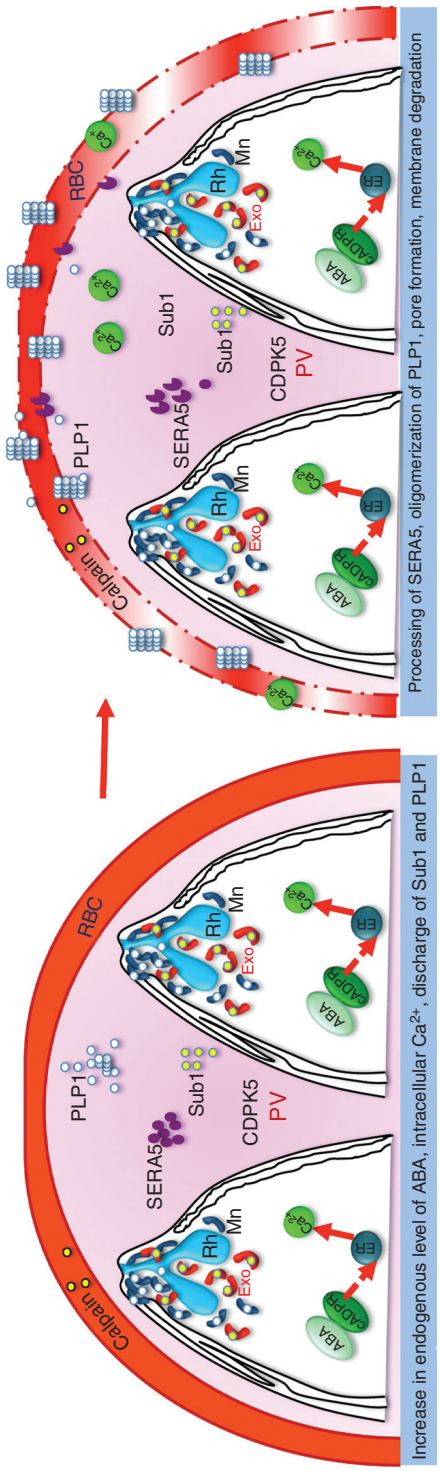

Figure 1. Regulation of merozoites' egress from host erythrocyte. An increase in abscisic acid (ABA) in schizonts leads to release of Ca^{2+} from the endoplasmic reticulum (ER) through the cADPR pathway. Increase in cytosolic Ca^{2+} triggers release of microneme (Mn) proteins such as PfPLP1 and exoneme (Exo) proteins such as PfSub1. Following secretion, PfPLP1 relocalizes to the limiting membranes and permeabilizes them to facilitate egress. Following its discharge to the parasitophorous vacuole (PV), PfSub1 mediates the processing of PfSERA5, leading to membrane degradation and egress.

the cytoplasm of the infected host cell until the schizont stage of parasite growth, when it relocated to the membrane following binding to Ca^{2+} and activation. Calpain-1 depletion from erythrocytes prevented parasite egress and led to growth arrest in the schizont stage, whereas reconstitution with recombinant calpain-1 restored normal growth development (Chandramohanadas et al. 2009). Human host cells treated with siRNA for host calpains (calpains 1 and 2) showed that it was required for *T. gondii* egress (Chandramohanadas et al. 2009).

ROLE OF APICAL ORGANELLE DISCHARGE IN REGULATION OF PARASITE EGRESS

Until recently, micronemes, the tubular shaped secretory organelles at the apical end of apicomplexan parasites, were thought to primarily contain adhesins that bind to cognate receptors on host cells during invasion. However, recently it has been shown that they also contain proteins that may be involved in parasite egress such as the pore-forming *T. gondii* perforin-like protein (TgPLP1) (Kafsack et al. 2009, 2010). It has been known for some time that microneme secretion is triggered by elevation of cytosolic Ca^{2+}. More recently, Ca^{2+} has also been implicated in regulating microneme secretion, which is required for parasite egress (Kafsack et al. 2009; Garg et al. 2013). Some of the molecular players in the calcium signaling pathway that play key roles in microneme secretion include TgCDPK1 and TgCDPK3, members of the calcium-dependent protein kinase family (Lourido et al. 2010). In a parallel study, using whole genome sequencing of *Toxoplasma* egress mutants, TgCDPK3 was found to be an essential factor for Ca^{2+}-induced egress from host cells. Because TgCDPK3 localizes to the periphery of the parasite, TgCDPK3 is proposed to be part of a signaling pathway that senses changes in the environment leading to egress (Lourido et al. 2012; McCoy et al. 2012). In case of *P. falciparum,* PfCDPK1 has been shown to play a role in microneme secretion (Bansal et al. 2013). In addition, although conditional deletion of PfCDPK5 results in a block in *P. falciparum* merozoite egress from mature schizonts, loss of PfCDPK5 does not impair RBC invasion (Dvorin et al. 2010). Whether PfCDPK5 is responsible for regulating release of an apical organelle that is required for egress remains to be determined.

The activation of cGMP-dependent protein kinase G (TgPKG) has also been shown to induce microneme secretion and egress. Compound 1, a selective apicomplexan PKG inhibitor, was used to establish the role of TgPKG in microneme secretion and egress (Wiersma et al. 2004). Treatment of *P. falciparum* schizonts with compound 1 results in a block in schizont rupture, which does not occur when transgenic parasites with a gatekeeper mutation are treated with compound 1 (Taylor et al. 2010; Collins et al. 2013). Phosphoproteomic analysis of merozoites with and without treatment with Compound 1 suggests that a cGMP-dependent kinase serves as a signaling hub that regulates key processes in invasion and egress of *P. falciparum* merozoites (Alam et al. 2015). Interestingly, in contrast to PfCDPK5 knockdown parasites, Compound 1-treated merozoites were not invasive (Dvorin et al. 2010). Collectively, these findings describe the complex signaling pathways that regulate egress and suggest that the different pathways cross talk with each other enabling the parasite to respond to multiple signals to orchestrate a timely escape from host cells.

MOLECULAR PLAYERS FOR ERYTHROCYTE INVASION BY *Plasmodium* spp. MEROZOITES

Following egress and release from mature schizonts, *Plasmodium* merozoites go on to invade uninfected erythrocytes to initiate another round of intracellular replication. Erythrocyte invasion by *Plasmodium spp.* merozoites has been studied extensively by live cell imaging and electron microscopy (Dvorak et al. 1975; Aikawa et al. 1978; Riglar et al. 2011; Hanssen et al. 2013). After attachment to erythrocytes, merozoites reorient and develop a tight junction between their apical ends and target erythrocytes (Aikawa et al. 1978; Besteiro et al. 2011; Riglar et al 2011). These steps are mediated by multiple interactions between parasite

protein ligands and host receptors (Gaur and Chitnis 2011; Cowman et al. 2012). The identity of host receptors involved in erythrocyte invasion came initially from studies that used genetically deficient erythrocytes or enzyme-treated erythrocytes for invasion studies. The first report on essentiality of a molecular interaction between *Plasmodium* spp. merozoites and erythrocytes during invasion was based on the observation that Duffy negativity in West Africa was associated with the absence of *P. vivax* infection (Miller et al. 1976). It was shown that *P. vivax* is dependent on interaction with the Duffy blood group antigen (also known as Duffy antigen receptor for chemokines [DARC]) to establish a blood-stage infection in humans (Miller et al. 1976; Horuk et al. 1993). However, recent studies have identified *P. vivax* strains in Kenya, Brazil, and Madagascar that can infect Duffy-negative individuals suggesting that *P. vivax* may invade using alternative invasion pathways (Ryan et al. 2006; Cavasini et al. 2007; Ménard et al. 2010). In case of *P. falciparum*, glycophorins A, B, C (GPA, GPB, GPC) were identified as receptors for invasion (Gaur and Chitnis 2011). However, no human erythrocyte lacking an individual receptor has been found to be completely refractory to invasion by *P. falciparum* indicating redundancy in invasion pathways. Following identification of host receptors, a family of erythrocyte-binding proteins (EBPs) that includes *P. vivax* and *P. knowlesi* Duffy-binding proteins (PvDBP and PkDBP) and *P. falciparum* erythrocyte-binding antigens, EBA-175, EBA-140, EBA-181, and EBL-1, were identified (Gaur and Chitnis 2011). GPA, GPB, and GPC have been shown to bind EBA-175, EBA-140, and EBL-1, respectively (Gaur and Chitnis 2011).

Another *P. falciparum* multigene family, which shares homology with *P. vivax* reticulocyte-binding proteins and binds erythrocytes, is the PfRH family of *P. falciparum* proteins (Gaur and Chitnis 2011). The PfRH protein family includes PfRH1a, PfRH1b PfRH2a, PfRH2b, PfRH4, and PfRH5. Genetic knockout studies have confirmed that PfRH2a/b and PfRH4 mediate sialic acid independent invasion pathways (Cowman et al. 2012). Complement receptor 1 (CR1) has recently been shown to serve as the erythrocyte receptor for PfRH4 (Tham et al. 2010). PfRH5 has also been shown to mediate invasion of Aotus erythrocytes by *P. falciparum* (Hayton et al. 2008). Basigin has been recently shown to be a receptor for PfRH5 binding (Crosnier et al. 2011). Other merozoite proteins implicated in invasion include thrombospondin-related anonymous protein (TRAP) homologs such as MTRAP and PTRAMP that contain the characteristic thrombospondin repeat (TSR) adhesive domains (Bartholdson et al. 2012; Siddiqui et al. 2013). Recent work has shown that the conserved apical membrane antigen-1 (PfAMA1) in complex with the rhoptry neck proteins (PfRON2 and PfRON4) localizes to the junction (Richard et al. 2010; Besteiro et al. 2011; Srinivasan et al. 2011). The exported PfRON2 protein is inserted into the erythrocyte membrane and interacts with secreted PfAMA1 during invasion. Other invasion-related proteins include two protein complexes, the high-molecular-weight (HMW) complex composed of RhopH1, RhopH2, and RhopH3 and the low-molecular-weight (LMW) complex composed of RAP1, RAP2, and RAP3. Apart from these adhesins and their interacting parasite ligands, *Plasmodium* spp. merozoites have a conserved molecular machinery for motility, which comprises of a central actin–myosin motor located in the pellicle of the parasite that is linked with both a surface adhesin and the inner membrane complex (IMC) and is likely to drive the invasion process (Sharma and Chitnis 2013). Recent work has shown that PfRON2-PfAMA1 interaction may not be essential for junction formation and there may be alternative pathways for invasion that are not dependent on the actin–myosin motor (Bargieri et al. 2014).

MOLECULAR SIGNALS THAT REGULATE APICAL ORGANELLE DISCHARGE DURING INVASION

As discussed above, many of the parasite proteins that bind erythrocyte receptors to mediate different steps in the invasion process are localized to the micronemes and rhoptries and must be translocated to the merozoite surface in a

regulated manner to enable receptor engagement and invasion. For example, PfAMA1 and PfEBA175 are localized in the micronemes, whereas PfRON2, and PfRON4 are localized in the rhoptry neck and PfRH2, PfRH5, PfRAP1-3, and PfRhop1-3 are localized in the rhoptry bulb. The timely discharge of microneme and rhoptry proteins is essential for successful invasion by *P. falciparum* merozoites (Singh et al. 2010; Riglar et al. 2011; Hanssen et al. 2013).

What are the external signals and internal signaling pathways that mediate discharge of microneme and rhoptry proteins in *Plasmodium* merozoites and *Toxoplasma* tachyzoites during invasion? Studies with *T. gondii* tachyzoites first revealed that the levels of free cytosolic Ca^{2+} are high in tachyzoites during the process of gliding and are restored to basal levels following attachment to target cells during invasion (Carruthers and Sibley 1999; Carruthers et al. 1999; Lovett et al. 2002; Moreno and Docampo 2003). Similarly, cytosolic Ca^{2+} levels are high in *P. falciparum* merozoites and are restored to basal levels following attachment to target erythrocytes during invasion indicating that Ca^{2+} may regulate processes such as vesicular secretion during invasion (Singh et al. 2010). Indeed, treatment of *P. falciparum* merozoites with A23187 triggers an increase in cytosolic Ca^{2+} and secretion of microneme proteins such as PfAMA1 and PfEBA-175 but has no effect on secretion of rhoptry proteins (Singh et al. 2010). Importantly, the Ca^{2+} ionophore, A23187, induces microneme secretion in absence of extracellular Ca^{2+}, which indicates that Ca^{2+} from intracellular stores is sufficient for secretion of microneme proteins (Singh et al. 2010). Ethanol and other short-chain alcohols elevate cytosolic Ca^{2+} in *T. gondii* tachyzoites to stimulate microneme secretion (Carruthers and Sibley 1999; Carruthers et al. 1999). Treatment of tachyzoites with thapsigargin, which inhibits sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and blocks pumping of Ca^{2+} from the cytosol back to the endoplasmic reticulum (ER), increases cytosolic [Ca^{2+}] levels and triggers secretion of microneme proteins. In contrast, chelation of cytosolic Ca^{2+} by treatment of *T. gondii* tachyzoites or *P. falciparum* merozoites with BAPTA-AM inhibits microneme release and invasion of host cells (Singh et al. 1990; Carruthers and Sibley 1999; Carruthers et al. 1999). Ca^{2+} is stored in organelles such as the ER, mitochondria, and acidocalcisomes in apicomplexan parasites (Garcia 1999; Alleva and Kirk 2001). Treatment of *T. gondii* tachyzoites with ethanol raises levels of intracellular inositol triphosphate (IP_3), which trigger Ca^{2+} release by binding to IP_3-receptor gated Ca^{2+} channels (IP_3R) on the ER. Furthermore, xestospongin C, an IP_3R antagonist, inhibits ethanol-triggered release of Ca^{2+} and microneme secretion in *T. gondii* suggesting the presence of IP_3R (Lovett et al. 2002). The presence of PLC has been shown in *T. gondii* and PLC transcripts have been detected in *P. falciparum* blood-stage parasites. Moreover, an inhibitor of PLC has been shown to block Ca^{2+} release in *T. gondii* and *P. falciparum* providing further evidence for the presence of an IP_3-mediated pathway for Ca^{2+} release in these parasites (Carruthers and Sibley 1999; Carruthers et al. 1999; Lovett et al. 2002; Moreno et al. 2003; Vaid et al. 2008; Singh et al. 2010). Treatment of *T. gondii* with ryanodine or caffeine, which are agonists of the ryanodine receptor (RyR)-gated Ca^{2+} channel on ER, also leads to an increase in cytosolic Ca^{2+} and microneme secretion (Lovett et al. 2002). The ER thus appears to be the primary intracellular store from which Ca^{2+} is mobilized. Endogenous cyclic ADP ribose (cADPR), which can bind RyR to induce calcium release, was detected in *T. gondii* extracts suggesting that it may also play a role as a second messenger to regulate calcium levels and microneme secretion (Chini et al. 2005). Moreover, treatment of *T. gondii* tachyzoites with RyR antagonists, 8-bromo-cADPR and ruthenium red, blocks microneme secretion (Chini et al. 2005). Although use of modulators has provided pharmacological evidence for the presence of both IP_3R and RyR in both *P. falciparum* and *T. gondii*, the genes encoding IP_3R and RyR have not yet been identified.

The increase in cytosolic [Ca^{2+}] levels activates *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) and *T. gondii* CDPK1

(TgCDPK1), which play central roles in orchestrating many of the processes required for invasion of host cells, including microneme release and the activation of the actin–myosin motor (Green et al. 2008; Kato et al. 2008b; Lourido et al. 2010; Bansal et al. 2013). Recently, a screen for molecules required for invasion of host cells by *T. gondii* identified a DOC2 protein that is required for microneme secretion, presumably by mediating Ca^{2+}-dependent vesicular docking activity (Farrell et al. 2012). The *P. falciparum* ortholog of this protein is also required for the invasion of red blood cells and release of micronemes (Farrell et al. 2012). Independent of Ca^{2+} release, chemical genetic and phosphoproteomics evidence suggests that cyclic guanosine monophosphate (cGMP) and the cGMP-dependent kinase, PKG, also play an essential role in microneme release and invasion (Moon et al. 2009; Alam et al. 2015).

What might be the external signals that trigger secretion of microneme and rhoptry proteins during invasion? We have shown that exposure of *P. falciparum* merozoites to a low-$[K^+]$ environment as found in blood plasma can serve as a natural signal that triggers an increase in cytosolic $[Ca^{2+}]$ through the PLC pathway leading to secretion of microneme proteins such as PfEBA175 and PfAMA1 to the merozoite surface (Singh et al. 2010). Following translocation to the merozoite surface, binding of EBA175 to GPA restores basal $[Ca^{2+}]$ levels and triggers release of rhoptry proteins such as PfRH2b, Clag3.1, and PfTRAMP (Singh et al. 2010; Siddiqui et al. 2013). Secretion of microneme and rhoptry proteins to the merozoite surface is thus a two-step process as shown in Figure 2. Microneme proteins have also been shown to play a role in rhoptry secretion in *T. gondii*. Deletion of microneme protein MIC8 resulted in a block in rhoptry secretion and parasite growth (Kessler et al. 2008). The cytoplasmic domain of microneme proteins was implicated in playing a role to trigger rhoptry secretion (Kessler et al. 2008).

The pathway by which a low-$[K^+]$ environment triggers an increase in cytosolic $[Ca^{2+}]$ in *P. falciparum* merozoites is regulated by another ubiquitous second messenger, namely, 3′-5′ cyclic adenosine monophosphate (cAMP) (Dawn et al. 2014). The transfer of *P. falciparum* merozoites to a low-$[K^+]$ medium activates the parasite carbonic anhydrase (PfCA), which produces H^+ ions and bicarbonate (HCO_3^-) to balance cytosolic pH (Dawn et al. 2014). Increase in cytosolic bicarbonate levels activates bicarbonate-sensitive soluble adenylate cyclase, PfACβ leading to an increase in cAMP levels, which activates protein kinase A (PfPKA) as well as the cAMP responsive GTP exchange protein called exchange protein activated by cAMP (PfEPAC) (Fig. 2) (Dawn et al. 2014). In other eukaryotic cells, activated EPAC transfers GTP to Rap1 GTPase, which then activates PLC (Gloerich and Bos 2010). Use of specific agonists as well as inhibitors has shown that the EPAC pathway for activation of PLC following an increase in cAMP is present and is responsible for the increase in $[Ca^{2+}]$ when merozoites are exposed to a low-$[K^+]$ environment following egress from schizonts. In addition to regulating levels of free $[Ca^{2+}]$ in merozoites, cAMP also directly regulates microneme secretion by activating PKA (Dawn et al. 2014).

A recent study describes a different mechanism for elevation of cytosolic $[Ca^{2+}]$ in merozoites leading to microneme release. This study suggests the interaction of rhoptry protein PfRh1 to host RBCs triggers a surge in cytosolic $[Ca^{2+}]$ levels in merozoites leading to release of microneme proteins. However, this model does not explain what triggers the release of PfRh1 from the rhoptries to the merozoite surface in the first place and implies that rhoptry secretion may precede microneme secretion (Gao et al. 2013).

The identification of some of the key players involved in signaling pathways leading to microneme and rhoptry discharge during invasion provides novel targets for intervention. For example, targeting PfCDPK1 with small molecule inhibitors will block release of key invasion-related microneme proteins to inhibit blood-stage parasite growth (Bansal et al. 2013). Given that enzymes such as PfCDPK1, which contain both the calmodulin-like Ca^{2+}-binding domain and kinase domain, are of plant origin and are not found in mammalian cells suggests that it should be possible to design specific inhibitors

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Figure 2. Model for cyclic adenosine monophosphate (cAMP) and Ca^{2+}-mediated signaling pathways that regulate apical organelle discharge in *Plasmodium falciparum* merozoites. Exposure of *P. falciparum* merozoites to a low-K^+ environment as present in blood plasma leads to production of H^+ and HCO_3^- ions by carbonic anhydrase (CA) to maintain pH. HCO_3^- ions activate soluble adeylate cyclase (PfACβ) leading to increase in levels of cAMP. Elevation of cAMP activates its downstream effectors, protein kinase A (PKA) and exchange protein activated by cAMP (EPAC). PKA plays a direct role in regulating microneme secretion. EPAC activates Rap1 GTPase by transferring GTP to Rap1. Rap1-GTP activates phospholipase C (PLC) leading to an increase in cytosolic Ca^{2+} levels, which leads to activation of calcium-dependent protein kinase 1 (PfCDPK1) and secretion of microneme proteins such as EBA175. Engagement of EBA175 with its receptor glycophorin A (GlyA) restores basal $[Ca^{2+}]$ levels and triggers discharge of rhoptry proteins such as PfClag3.1. Mn, Micronemes; Rh, rhoptries.

that block activation of PfCDPK1 following binding to Ca^{2+}. Similarly other *P. falciparum* enzymes such as carbonic anhydrase (PfCA) or adenylate cyclase (PfACβ) could also be targeted to block production of second messengers required for transduction of signals that trigger microneme release to block blood-stage growth. The signaling pathways that lead to release of rhoptry proteins remain to be defined. Targeting both signaling pathways that are involved in microneme and rhoptry release may provide synergy to efficiently block erythrocyte invasion by apicomplexan pathogens.

FUTURE PERSPECTIVES

Significant progress has been made toward understanding the molecular basis of key steps in the biology of blood-stage malaria parasites such as RBC invasion and merozoite egress following replication in schizonts. As we identify the key parasite ligands involved in invasion, it is clear that it is unlikely that a single merozoite antigen will hold the key to the invasion process. Instead, we have learned that the invasion process is complex and has multiple steps with redundancies built into each step. So, there will be no key parasite ligand that can be targeted to block invasion. It is more likely that we will find combinations of antigens that are critical for invasion and yield synergy to block invasion with high efficiency when targeted together with a combination of specific antibodies. Such combinations may yield high rates of growth inhibition across diverse parasite strains and may provide the basis of highly effective blood-stage vaccines. Such vaccines may have an impact not only on blood-stage parasitemia but also on gametocyte densities leading to a reduction in transmission potential. Such vaccines that interrupt malaria transmission (VIMT) may be useful in elimination strategies in endemic regions in addition to providing protection against malaria.

The regulated secretion of key effector molecules from merozoite apical organelles plays an important role in the complex processes of host cell invasion and egress. A clear understanding of the complete signaling cascades that regulate apical organelle secretion is likely to reveal targets for small molecule inhibitors that can block invasion or egress. Such targets may provide attractive points for intervention to block blood-stage parasite growth. Some of the signaling pathways may be common to different stages. For example, development of gametes from gametocytes is also regulated by calcium and requires secretion of effectors such as perforin-like protein PfPLP2 for gamete egress. The signaling pathways that regulate processes such as gametocyte activation and gamete egress are not understood. If the signaling pathways that regulate steps such as vesicular secretion leading to egress of sexual-stage parasites are similar to those in blood stages, it may be possible to target both blood-stage growth and malaria transmission. Such interventions can not only protect against malaria but also assist in elimination of malaria by reducing malaria transmission.

CONCLUDING REMARKS

During blood-stage infection *Plasmodium* spp. merozoites repeatedly invade, multiply, exit, and reinvade host RBCs. The key steps of exit from mature schizonts and reinvasion of RBCs by merozoites requires timely regulation of key cellular processes such as apical organelle discharge, which is regulated by cAMP and Ca^{2+}-dependent signal transduction pathways. The internal and external signals that regulate levels of second messengers such as cAMP and Ca^{2+} have been identified and some of the key players of the signaling pathways are known. A complete understanding of the signaling pathways that regulate the process of apical organelle secretion will enable the design of novel intervention strategies that block parasite egress or invasion to block blood-stage parasite growth. Some of the signaling pathways that regulate processes such as vesicular discharge in the blood stage may be common to other stages as well. It may thus be possible to target pathways that are common to both the blood and sexual stages to provide protection against malaria as well as block malaria transmission to eventually eliminate malaria.

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