Trends in Parasitology

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Review

Important Extracellular Interactions between *Plasmodium* Sporozoites and Host Cells Required for Infection

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Malaria is an infectious disease, caused by *Plasmodium* parasites, that remains a major global health problem. Infection begins when salivary gland sporozoites are transmitted through the bite of an infected mosquito. Once within the host, sporozoites navigate through the dermis, into the bloodstream, and eventually invade hepatocytes. While we have an increasingly sophisticated cellular description of this journey, our molecular understanding of the extracellular interactions between the sporozoite and mammalian host that regulate migration and invasion remain comparatively poor. Here, we review the current state of our understanding, highlight the technical limitations that have frustrated progress, and outline how new approaches will help to address this knowledge gap with the ultimate aim of improving malaria treatments.

Highlights

Cellular descriptions of sporozoite behaviors and host cell interactions are increasingly well defined; however, signals triggering sporozoite behavioral switches remain unknown.

The molecular basis of sporozoite–host recognition is poorly characterized due to technical challenges that have limited progress.

New genetic and biochemical techniques are addressing some of these challenges and have identified extracellular sporozoite–host interactions involved in host infection.

Using these new tools to understand the molecular basis of host–sporozoite interactions will help to improve antimalarial treatments and vaccines.

Sporozoites and Malaria Infection

Malaria is an infectious disease caused by *Plasmodium* parasites, of which *P. falciparum* causes most morbidity and mortality. Around 40% of the global population is considered at risk of infection, and almost half a million deaths were attributed to malaria in 2016 [1]. *Plasmodium* parasites are unicellular eukaryotes in the phylum Apicomplexa. They have motile extracellular forms called '-zoites' that can actively invade host cells where they multiply. Infection is initiated when an infected female anopheline mosquito searches for blood and transmits *Plasmodium* sporozoites into the dermis of the host. Sporozoites move within the skin in apparently random directions to locate and then enter blood vessels, and from there they are passively transported in the bloodstream and rapidly reach the liver. Sporozoites leave the circulation by crossing the liver sinusoidal capillary barrier and enter the liver parenchyma where they traverse through several hepatocytes before productively invading a cell and developing into a liver-stage form. Here, they multiply into thousands of merozoites which are released into the bloodstream to invade erythrocytes and initiate the blood stage of infection. The asexual blood stage involves the rapid and iterative invasion of erythrocytes and is the symptomatic part of the life cycle.

Sporozoites are considered an attractive target for therapeutic intervention, particularly for vaccines. This is firstly because sporozoites constitute a bottleneck in the parasite life cycle, with only around 10 to 100 sporozoites transmitted by an infected mosquito bite [2]. Secondly, the extracellular sporozoites are exposed to host antibodies for minutes to hours compared with just minutes for the blood stages [3]. Finally, the sporozoite stage is asymptomatic, and so targeting sporozoites could prevent clinical disease. Molecular interactions between sporozoites and the host are likely to be important for the parasite's journey to the liver, and these interactions would be ideal vaccine targets. Indeed, subunit vaccines based on sporozoite surface proteins and attenuated whole sporozoites show some protection in animal models and humans [4].

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The development of *in vitro* sporozoite culture methods, advances in microscopy techniques, and the use of tractable animal models of malaria have resulted in a detailed cellular description of how sporozoites interact with their host; however, we know little at the molecular level, leaving many questions unanswered. How do sporozoites recognize the different cell types that act as signposts along its journey to the hepatocyte? Are there host cues that instruct sporozoites to switch behavior between cell traversal and invasion? What are the host–parasite receptor–ligand interactions involved in guiding sporozoites? With the advent of new techniques and technologies, we are now starting to uncover some of these important sporozoite–host interactions. This review focuses on sporozoite behaviors and their cellular and extracellular molecular interactions with host cells.

Cellular Architecture of Sporozoites

Sporozoites are slender, crescent-shaped cells that measure approximately 10–12 μm by 1–2 μm. They develop in oocysts on the mosquito midgut wall and are released into the hemocoel where they are carried throughout the open circulatory system of the mosquito, with ~20% ultimately invading salivary glands. Oocyst and salivary-gland sporozoites differ, with the latter competent for infection of the mammalian host, a phenotype accompanied by changes in the sporozoite cytoskeleton [5], motility [6,7], and surface proteins [8]. Transcriptomic and proteomic studies of oocyst and salivary gland sporozoites have identified transcriptional changes associated with maturation [9], and described a repertoire of ~2000 proteins expressed by mature sporozoites [10]. Similar to other motile zoites, sporozoites have distinct cell polarity, specialized motility apparatus, and apical secretory organelles called micronemes, rhoptries, and dense granules that have separate yet complementary roles in motility and invasion [11] (Figure 1A, Key Figure). The sporozoite surface is densely covered by the **circumsporozoite protein** (**CSP**) (see Glossary), an essential and multifunctional protein that is likely linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [12]. The **thrombospondin-related anonymous protein** (**TRAP**), a micronemal protein secreted to the sporozoite surface during motility, likewise plays a critical role in motility [13]. Targeted proteomic analysis of the surface of mature sporozoites has further identified several dozen surface proteins in addition to CSP and TRAP that might interact with host factors [14]. Alongside this, several other important secreted or sporozoite surface proteins such as CelTOS, P36, and P52 have also been characterized. As outlined below, ongoing genetic, biochemical, and molecular analyses are helping to link this knowledge of the sporozoite's cellular architecture to specific sporozoite behaviors and their underlying molecular effectors.

Cellular Behaviors of Sporozoites

Sporozoites display several specialized behaviors on their journey to the liver. Sporozoites move by **gliding motility**, a type of substrate-based motility characterized by a lack of cilia or flagella, and the absence of any overt change in cell shape [15]. After inoculation into the skin, sporozoites must breach several cell barriers to reach the hepatocyte. This, in part, relies on a process known as **cell traversal**, which enables sporozoites to cross host cell membranes and cell barriers. Using motility and cell traversal, sporozoites ultimately reach and invade a hepatocyte (Figure 1A,B). This **cell invasion** is characterized by the formation of a parasitophorous vacuole: an intracellular membrane within which the parasite resides and develops until the completion of the liver stage.

Sporozoite Gliding Motility

Sporozoites are among the fastest eukaryotic cells, moving at 1–3 μm per second *in vitro* and *in vivo*. In the mammalian host, motility is essential for sporozoite exit from the dermal inoculation site and subsequent hepatocyte invasion [13,16,17]. Critical to gliding motility are the TRAP family of transmembrane proteins. These proteins share several features in their domain

Glossary

AVidity-based EXtracellular Interaction Screen (AVEXIS): a method designed to detect low-affinity extracellular protein interactions that can be scaled to systematically screen many thousands of potential interactions. It has been used to identify novel endogenous interactions between receptor proteins displayed on cells of the same species and to identify host–pathogen interactions.

Cell invasion: a sporozoite behaviour that allows for entry into a host cell to establish an infection. It involves the formation of a parasitophorous vacuole membrane around the parasite that separates it from the host cell cytoplasm.

Cell traversal: a sporozoite behaviour that allows for transient passage through a host cell in order to migrate through cells or tissues and escape phagocytic destruction. As sporozoites move through and exit host cells they wound the host cell plasma membrane.

Circumsporozoite protein (CSP): a highly abundant, multifunctional protein that densely covers the surface of sporozoites. CSP is essential for sporozoite formation in the mosquito host and guiding the migration of sporozoites from mosquito to mammalian host. CSP is highly immunogenic, and is the target of the current leading anti-sporozoite vaccine.

Gliding motility: a type of substrate-dependent motility that does not involve changes to the cell shape, but instead relies on the parasite cytoskeleton and secreted cell surface proteins. It is required for sporozoite migration, cell traversal, and invasion.

Heparan sulfate proteoglycans (HSPGs): cell surface proteins on animal cells that contain linear sulfated glycosaminoglycan polysaccharides. HSPGs are a major constituent of the glycocalyx and are thought to be more highly sulfated on the surface of stellate cells in the liver and in the loose basement membrane of the liver called the Space of Disse.

Thrombospondin-related anonymous protein (TRAP): an abundant transmembrane protein that is secreted onto the sporozoite

Key Figure

Extracellular Interactions Involved in *Plasmodium* Sporozoite Migration and Invasion

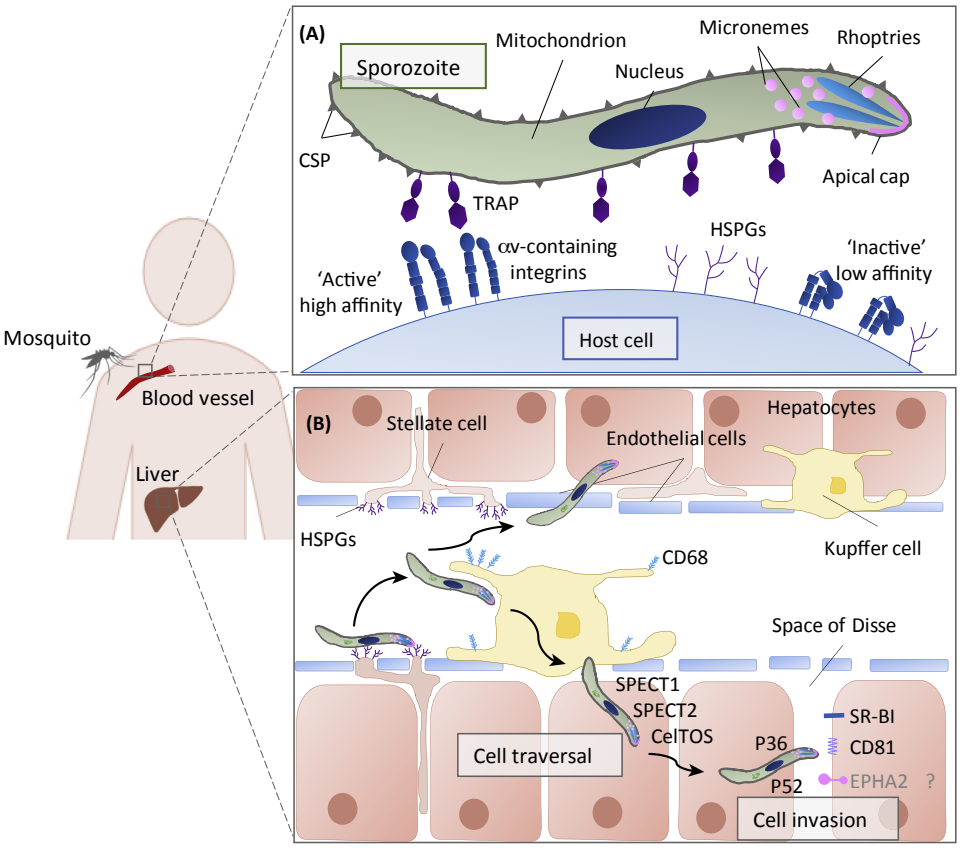

Figure 1. Sporozoites are deposited in the skin of the host when an infected mosquito takes a blood meal, and move within the dermis by an active process known as gliding motility. Sporozoites recognize and traverse the vascular endothelium to enter the bloodstream where they are transported to the liver. (A) The abundant sporozoite surface protein, thrombospondin-related anonymous protein (TRAP), which is essential for gliding motility, has been recently found to interact with αv-containing integrins in their extended active conformation displayed on the surface of host cells, and may be involved in cell host recognition events in the dermis. (B) Sporozoites are transported to the liver where they are arrested within the liver sinusoids: specialized capillary beds bordered by a highly porous epithelium which contain fenestrations that are clustered into structures known as sieve plates. Hepatocytes are not separated from the vascular endothelial cells by the usual basement membrane, but rather by a conspicuous gap known as the space of Disse that is occupied by stellate cells; the lumen of the sinusoid is patrolled by hepatic macrophages called Kupffer cells. Parasites are thought to be arrested in the liver by interactions between the abundant sporozoite surface protein circumsporozoite protein (CSP) and unusually high levels of sulfated heparan sulfate proteoglycans (HSPGs) displayed by stellate cells that protrude as tufts through the fenestrations in the sinusoid endothelium. The arrested sporozoite enters the liver parenchyma either by cell traversal of Kupffer cells, which appears to involve CD68, or by traversal of endothelial cells. Sporozoites then traverse through several hepatocytes, a process which requires the action of the sporozoite proteins SPECT1, SPECT2, and CelTOS. Eventually, sporozoites productively invade a hepatocyte, requiring the parasite proteins P36 and P52, and the host receptors SR-BI, and/or CD81, and potentially EPHA2.

surface during gliding motility. TRAP is a critical component of the sporozoite's motility machinery, serving to link intracellular cytoskeletal elements with extracellular substrates or ligands. It is essential for sporozoite motility and hence normal traversal and invasion.

architecture, including extracellular adhesion domains and a cytoplasmic domain that couples to an actinomyosin molecular motor [13]. To propel itself, the parasite must adhere to a substrate or cell to provide traction for displacement [15]. A study using reflection interference contrast microscopy (RICM) to examine the contact points between sporozoites and the substrate revealed that 'gliding motility' is in fact saltatory, involving the stepwise formation and release of discrete adhesion sites [18]. TRAP is the most critical TRAP family member, with targeted mutations of *trap* significantly affecting motility and infectivity [17,19], revealing the importance of gliding motility for these events.

Advances in microscopy and the accessibility of the skin enabled sporozoite gliding within the dermis to be visualized *in vivo*. Quantitative imaging of sporozoites from the rodent malaria model *Plasmodium berghei* in the mouse ear demonstrated that, within 1 h, approximately half of the sporozoites enter the blood (20%) or lymphatic (30%) vessels [16], with the rest remaining in the dermis for up to 7 h after transmission. Two distinct patterns of sporozoite motility were identified: within the dermis, they moved quickly and were displaced over relatively large distances, while within the vicinity of a blood vessel their movement was slower and the curvature of their path more constrained, a motility pattern that may optimize blood vessel contact [17]. This suggests that a specific, but unidentified host–parasite recognition event occurs at the blood vessels, initiating a change in sporozoite behavior, although further work is required to fully understand this critical process.

Sporozoite Cell Traversal

The ability of sporozoites to traverse host cells is important at several points along its journey from the site of deposition to the hepatocyte. Cell traversal was first described when *P. berghei* sporozoites were observed to transit through host macrophages and later hepatocytes and epithelial cells [20]. There is debate about whether cell traversal involves the formation of a transient parasitophorous vacuole or if it ruptures the host cell membrane [21]. Several parasite proteins involved in cell traversal have been identified, with knockout of genes encoding either SPECT1 (Sporozoite Protein Essential for Cell Traversal 1) [22] or SPECT2 [23,24] found to affect sporozoite traversal. The sequence similarities of SPECT2 with membrane pore-forming proteins and the ability of purified recombinant protein to disrupt host membranes suggest that SPECT2 functions by interacting with host phospholipids to perturb membranes [25]. The role of SPECT1, however, is less clear. In the liver, sporozoites cross the sinusoidal barrier mainly by traversing Kupffer cells: specialized macrophages that line the endothelial vessels of the liver sinusoids. SPECT1-deficient sporozoites are weakly infective *in vivo*, but regain normal infectivity if Kupffer cells are depleted [22,24]. A more recent study has shown that when endothelial cells are loaded with dye that is dispersed upon cell wounding, these cells can also be used by sporozoites to enter the liver parenchyma and that cell traversal enables the parasites to avoid phagocytic destruction by Kupffer cells (Figure 1B) [26]. Given the large size of Kupffer cells compared to endothelial cells, sporozoites are more likely to contact these cells and use their ability to traverse cells to avoid phagocytosis and successfully navigate entry into the liver [26]. In addition, the same mechanisms that are used to traverse cells in the mammalian host are likely to operate in the mosquito, and a small secreted sporozoite protein called CelTOS has been shown to be necessary for crossing the cytoplasm of both hepatocytes and the insect midgut epithelium [27]. The CelTOS crystal structure and ability of the recombinant protein to bind to lipids suggests that it may also act by helping to perturb the host cell membrane during traversal [28]. Together, these studies highlight important roles for cell traversal in transmission and infection that are independent of invasion.

Sporozoite Invasion

After crossing the sinusoidal barrier, sporozoites switch to an invasive state and invade hepatocytes with the concurrent formation of a parasitophorous vacuole membrane [29–

31]. Invasion involves the regulated secretion of parasite ligands from apical organelles to form a membranous connection, called a tight junction, between the sporozoite apical tip and the host cell, which opens up in a ring-like structure through which the sporozoite invades using the same molecular machinery that powers gliding motility [32]. The parasite surface proteins AMA1 and RON2 are thought to be essential for tight junction formation [33], but knockdown of AMA1 to undetectable levels in *P. berghei* had no effect on sporozoite invasion of hepatocytes [34]. This finding was later confirmed by complete knockout of AMA1 in *P. berghei* sporozoites [35]. In addition, two proteins which contain 6-cysteine domains, P36 and P52, have been implicated in hepatocyte invasion, although their precise roles are still unclear. *P. berghei* and *P. yoelli* sporozoites with targeted mutations in both *p36* and *p52* were able to traverse cells but did not commit to productive invasion [36]; however, *P. falciparum* sporozoites lacking both genes appeared to invade cells but were instead arrested during liver-stage development [37]. It is likely that P36 and P52 are required after invasion for proper formation of the parasitophorous vacuole [37], which protects the parasite from destruction once in the host hepatocyte. Thus, the molecules involved in sporozoite invasion of hepatocytes and their precise functions are still incompletely defined. As we will discuss, a small number of host cell surface proteins, such as CD81, SR-BI, and CD68, are now known to be important for sporozoite traversal and invasion, but exactly how these interact with specific parasite ligands is also still undetermined.

Switching between Behaviors

Major unanswered questions are how the sporozoite switches between different cellular behaviors during its migration from the dermis to the liver, and how the sporozoite distinguishes the liver from other host tissues. The most abundant protein on the surface of the sporozoite, CSP, is thought to sense the relatively higher level of sulfation of **heparan sulfate proteoglycans** (**HSPGs**) displayed by the stellate cells that protrude into the vascular lumen through fenestrations in the hepatic vascular endothelium [20,38] (Figure 1B). How sporozoites switch from a traversal to an invasive phenotype is also a subject of debate. Early work suggested that it was the traversal of hepatocytes that allowed the sporozoites to switch, either by increasing secretion of organelles required for invasion [39], or by increased exposure to intracellular potassium [40]. It was also suggested that wounded hepatocytes secrete hepatocyte growth factor, which, after interacting with its receptor on neighboring cells, made them more susceptible to infection [41]; however, this was found to apply only in *P. berghei* but not in *P. yoelii* or *P. falciparum* [42]. These theories are contradicted by the fact that mutant sporozoites deficient in cell traversal can still infect hepatocytes when Kupffer cells are depleted [22,24]. One model that does not require cell traversal to trigger the switch to invasion again invokes the presence of highly sulfated HSPGs within the liver, but to trigger the proinvasive proteolytic cleavage of CSP [30]; however, this too remains to be unequivocally demonstrated. Overall, the host molecular cues and how these are perceived by the sporozoite in the extracellular environment remain poorly defined.

Identifying Extracellular Host–Parasite Interactions

Extracellular recognition events are critical for host–pathogen interactions and for defining sporozoite cell and host tropisms; however, we know little about the host factors that act as molecular guide-posts for sporozoites. Given their importance for infection biology and their potential for therapeutic intervention, why is this so? Likely reasons include the limited amounts of sporozoite material which, by contrast to blood stages, cannot be expanded *in vitro*, and must be dissected from mosquito salivary glands. Furthermore, proteins that bridge the plasma membrane are typically biochemically intractable: their amphipathic nature makes them difficult to solubilize in their native conformation, and extracellular receptor–ligand interactions are often

highly transient such that they cannot withstand wash steps common to biochemical purification protocols [43]. These challenges could be tackled by using soluble recombinant proteins corresponding to ectodomains which often retain their receptor binding activity; however, a suitable protein expression system must be selected because these regions occupy an oxidizing environment and structurally critical post-translational modifications such as disulfide bonds must be formed correctly.

Since many of these challenges are not specific to parasites, recent advances in eukaryotic recombinant protein expression systems have enabled soluble versions of the ectodomains of *Plasmodium* ligands to be produced in biochemically active forms [44]. These can be used as binding probes to detect interactions with host tissues and molecules; however, so far, no widely accepted host–sporozoite interactions have been identified using standard biochemical approaches. Some success has been achieved using a bespoke protein interaction assay designed to detect transient extracellular protein interactions called **AVEXIS** (**AVidity-based EXtracellular Interaction Screen**) [45]. This method uses direct binding within libraries of recombinant proteins constituting the extracellular regions of host and parasite proteins in an ELISA-style assay. This has successfully identified host receptors for blood-stage *Plasmodium* ligands [46] and more recently identified αv-containing host integrins as a ligand for TRAP in sporozoites [47] (Figure 1A). One challenge with this approach is that the sporozoite interacts with many host cell types, making the library of recombinant proteins impractically large for most laboratories. Other approaches include screening cellular microarrays that have been transfected with cDNA constructs representing a large number of host receptors [48]. Again, such large collections of reagents are usually beyond the reach of most laboratories, but the recent development of cell-based genome-wide genetic loss-of-function screens provide the possibility of identifying host receptors without needing large reagent collections [49].

Important Sporozoite Surface Proteins and Known Host Interactions

The two most abundant proteins displayed at the surface of the sporozoite are CSP and TRAP. The functional role of both proteins has been investigated in detail.

CSP

The overall structure of CSP is conserved among *Plasmodium* species, with a central repeat region whose sequence varies between species, a C terminal type I thrombospondin repeat (TSR) adhesion domain, and a conserved five amino acid sequence N terminal to the repeats that serves as a proteolytic cleavage site [29]. Deletion of the *csp* gene results in a severe defect in sporozoite development [50], and studies indicate the TSR domain is likely involved in this process [51]. The efficiency of sporozoite salivary gland invasion and infection of the mammalian host critically depends on interactions between CSP and both mosquito and mammalian host tissues. During sporozoite migration, CSP is folded such that the TSR is masked, a conformation that enables sporozoites to efficiently navigate through tissues [17,29]. Once in the liver, a parasite protease cleaves CSP, leading to the removal of the N terminal third of the protein and exposure of the TSR [29,52]. The signal for this cleavage event is, in part, reliant upon sporozoite contact with HSPGs, and the resulting exposure of the TSR is associated with a switch from a migratory to an invasive phenotype [30].

The controlled exposure of CSP domains suggests that different portions of CSP may function in the recognition of host tissues; however, there remains much to be learnt about these events. Several studies have found that, in the mosquito, the CSP N terminus binds to the salivary glands [53,54], and inhibition of this leads to lower salivary gland infections [55,56]. Previous studies describing CSP binding to HSPGs in the mammalian host (described below) spurred

the search for similar molecules in the mosquito. HSPGs are indeed found on mosquito salivary glands [57] and these can mediate salivary gland invasion [58]; however, in their absence, other surface molecules compensate. This suggests that HSPGs may mediate initial adhesion to mosquito salivary glands via the N terminus of CSP, but that other interactions are involved; for example, a yet to be fully characterized CSP-binding protein on mosquito salivary glands has been described [54]. Other secreted or sporozoite surface proteins such as TREP [59], a member of the TRAP family, and MAEBL [8,60], are also essential for salivary gland invasion, although their binding partners have not been identified.

In the mammalian host, a considerable body of work has demonstrated that CSP binds to HSPGs. Initial studies demonstrated that recombinant CSP bound to hepatocyte HSPGs [61] and that this was more important under shear stress conditions [38], suggesting that it may mediate localization of sporozoites to the liver but not hepatocyte invasion *per se*. Indeed, *in vitro* studies demonstrated that heparin had a negligible effect on sporozoite invasion into hepatocytes under static conditions [38,62]. The identity of the heparin-binding domain of CSP remains controversial. Initial studies using peptides representing portions of the TSR in binding competition assays suggested that the TSR had heparin-binding activity [61]; however, a recent crystal structure of the CSP TSR was described and this protein had no heparin-binding activity [63], suggesting that previous data with peptides did not reflect the activity of the native protein. In subsequent studies, peptides and recombinant protein representing the N terminus of CSP were shown to have high-affinity heparin-binding activity [64], raising the possibility that the CSP N terminus binds to hepatic HSPGs, a scenario consistent with what we currently know of CSP domain conformation. Since CSP densely coats the sporozoite, one can imagine that CSP cleavage and subsequent exposure of an array of TSRs would make for a very adhesive sporozoite that could, upon binding to a yet-to-be-identified receptor, provide the signal for it to switch to an invasive phenotype. How can hepatic HSPGs provide a signal to sporozoites that they are in the liver when HSPGs are found in many tissues and cell types? Previous studies found that it was only the highly sulfated HSPGs that bound to CSP, and the degree of sulfation, but not a specific pattern of sulfation or specific saccharide, that was critical for sporozoite binding and induction of CSP cleavage [30,38]. In the mammalian host, the only highly sulfated HSPGs exposed to the circulation are in the liver sinusoids, explaining how they can account for the specific arrest of sporozoites in this organ.

TRAP

TRAP is a type I transmembrane protein containing a von Willebrand factor A (VWA) domain and a TSR domain in its extracellular region. It is the founding member of a family of TRAP-like proteins that are expressed in sporozoites (such as TLP, TREP), and other invasive *Plasmodium* stages (MTRAP and CTRP) [32]. TRAP is mainly localized to micronemes [65,66] but is trafficked to the surface and shed from the posterior end of gliding sporozoites [19]. TRAP-deficient *P. berghei* parasites exhibit multiple defects: they are unable to glide *in vitro*, and are significantly impaired in their ability to invade mosquito salivary glands and infect rodent hosts. These and other data provide evidence that TRAP is an essential component of the sporozoite gliding motility and invasion machinery [13]. Consistent with this, parasites with mutations in the cytoplasmic region of TRAP exhibit abnormal gliding and are unable to infect mice or invade hepatocytes *in vitro* [67].

Early work suggested that TRAP interacted directly with HSPGs, identifying a conserved motif within the TSR domain that is important for TRAP binding to glycans in plate-based assays and on hepatocytes [68]. Genetic studies of TRAP in *P. berghei* have further dissected the roles of the VWA and TSR domains, with both domains being found to be important in host cell

invasion, but not gliding motility [69]. The structure of the TRAP ectodomain was recently solved and broadened our understanding of TRAP function since it found that TRAP adopted at least two conformations: a compact 'closed' form and an extended 'open' conformation [70]. One possibility is that binding of TRAP to a host receptor triggers this conformational change, generating additional force that is transmitted along the extended structure. Such a phenomenon might provide insight into the mechanism for the proposed saltatory 'stick and slip' model of gliding motility [18].

Our recent study identified αv-containing integrins as host receptors for *P. falciparum* TRAP [47]. The interaction between TRAP and integrin was shown to be high affinity, and binding was enhanced by manganese ions, a known integrin activator, demonstrating that TRAP may bind preferentially to integrins in their activated ligand binding state (Figure 1A). This preference may restrict TRAP-mediated interactions to clusters of activated integrins such as at focal adhesion sites. There was no evidence that this interaction was involved in hepatocyte invasion, but *P. falciparum* sporozoites did glide more rapidly and with a greater displacement in integrin-deficient mice. This suggests that the role for this interaction might be to act as 'stop' signal in the dermis in response to a localized signal from the host [47]. However, further work is required to more fully understand the role of this interaction in sporozoite migration and infection.

Other Host Proteins Important for Sporozoite Infections

Erythrocyte invasion by *Plasmodium* merozoites provide an important paradigm for understanding molecular interactions required for host cell invasion. Here, we know that ~50 different cell surface or secreted parasite proteins are involved [11] and have begun to delineate the order in which they are deployed to interact with host receptors during the multistep invasion process [71]. There is every reason to believe that traversal and invasion of hepatocytes by sporozoites is equally complex, but so far, only a handful of host receptor proteins have been identified, including CD81, SR-B1, CD68, and potentially EPHA2.

In a remarkable finding, *Cd81*-deficient mice were shown to be refractory to *P. yoelii* liver-stage but not blood-stage infection, leading the authors to conclude that host CD81 was required for both *P. yoelii* and *P. falciparum* sporozoites to invade hepatocytes [72]. CD81 belongs to the class of tetraspanin receptors, so named because they traverse the membrane four times [73]. CD81 and other tetraspanins, such as CD9, have documented roles in manipulation of membranes and are involved in membrane fusion events including fertilization [74] and viral infections [75]. Host CD81 was later shown to be necessary for the discharge of *P. yoelii* sporozoite rhoptry contents and may be the source of the host signal that switches sporozoites from cell traversal to invasion, since parasites preferentially traverse cells lacking CD81, but require it for productive invasion [76,77]. Because of these cell-type specific functions but broad expression pattern, CD81 is thought to be required for the organization of other tissue-restricted cell surface proteins, and/or to stabilize intermediates in membrane curvature needed for rhoptry discharge and parasitophorous vacuole formation. However, while undoubtedly critical for both *P. yoelii* and *P. falciparum* sporozoite invasion [72], the precise role of CD81 in invasion is currently unknown.

Using clues that the host lipoprotein clearance pathway was important for liver infection [78], the scavenger receptor BI (SR-BI) was found to have dual roles in *P. berghei* and *P. falciparum* sporozoite invasion and development within hepatocytes [79,80]. The amount of CD81 detectable at the cell surface was significantly decreased in cells lacking SR-BI, possibly due to a reduction in cellular cholesterol levels. In light of the recent crystal structure of CD81 that showed a cholesterol molecule bound within the intramembranous pocket formed

by the four transmembrane-spanning helices, these data could suggest that this is an important feature of CD81 and hence SB-RI function [73].

Searching for host surface proteins important for sporozoite infection, Cha and colleagues [81] selected peptide-displaying phages from a large library that bound intact Kupffer cells and prevented *P. berghei* sporozoite entry. They identified the target of these entry-blocking peptides as CD68: a large heavily O-glycosylated type I membrane protein mainly localized to the endosomes and lysosomes of macrophages and monocytes. *Cd68*-deficient mice exhibited a strong but incomplete reduction in *P. berghei* liver infection, suggesting an important but redundant role for CD68 and Kupffer cells in the exit of sporozoites from circulation, consistent with other sporozoite routes to the liver [26]. Host CD68, therefore, appears to play at least some role in sporozoite traversal prior to hepatocyte invasion.

Exploiting differences in susceptibility of different mouse strains and hepatocytes to *P. yoelii* infection, the Ephrin receptor A2 (EPHA2) was implicated in sporozoite invasion of hepatocytes, with higher levels of EPHA2 expression correlated with increased invasion by *P. yoelii* and *P. falciparum* sporozoites [82]. The same study also found that *EphA2*-deficient mice were less susceptible to infection with *P. yoelii* sporozoites, and that antibodies to EPHA2 could markedly inhibit sporozoite invasion *in vitro*. Together with other data, these findings supported the conclusion that EPHA2 was important for sporozoite invasion [82]. Recent studies using siRNA to knockdown EPHA2 expression in hepatocytes and similar antibody inhibition studies, however, did not support a role for EPHA2 in hepatocyte invasion for either *P. yoelii* or *P. berghei* [83]. Indeed, EPHA2 knockdown appeared to have no clear effect on sporozoite invasion regardless of the hepatocyte source, and antibodies to EPHA2 showed no greater ability to inhibit sporozoite invasion compared to the control [83]. In light of these conflicting results, the precise role of EPHA2 in sporozoite invasion remains an open question.

For all of these host receptors, the role in sporozoite infection would be greatly clarified by identifying the parasite molecules they interact with. While there are data to suggest that EPHA2 might interact with P36 [82], so far there has been no conclusive evidence for any of these receptors interacting directly with a parasite protein. Clarifying the molecular events involved in host–sporozoite interactions is further complicated because experiments suggest, as might be expected, that different species of *Plasmodium* use different host factors. For example, CD81 is important for the invasion of hepatocytes by *P. yoelii* and *P. falciparum*, but is not required by *P. vivax*, and is redundant with SR-BI for *P. berghei* [36]. Similarly, SR-BI is required for *P. vivax* but not *P. falciparum* or *P. yoelii* [36]. These molecular differences make extrapolating paradigms established in experimentally tractable rodent infection models to the *Plasmodium* species that cause disease in humans difficult.

Concluding Remarks

We are far from having a good molecular understanding of how sporozoites recognize and interact with mammalian host cells (see Outstanding Questions). Progress has been hard won because of experimental challenges associated with manipulation of sporozoites and membrane-embedded cell-surface proteins. Sporozoites can be produced only in mosquitoes, and the relatively modest numbers that can be obtained, together with the inevitable mosquito contamination, have significantly hindered our ability to cleanly define host–pathogen interactions of this stage of the *Plasmodium* life cycle. Future advances will rely on the development and application of new techniques and models, including *in vivo* imaging technologies, humanized mice, and methods to identify extracellular protein interactions. There are many reasons to be optimistic, particularly in light of recent genome-scale genetic techniques to

Outstanding Questions

What are the molecular identities of the host–sporozoite receptor–ligand interactions responsible for cellular recognition events within the host?

What are the interactions that signal to the sporozoite to become motile and to switch to an invasive state?

Specifically, what is the identity of the interactions that are responsible for:
- providing a substrate for sporozoite gliding;
- specific recognition of vascular endothelial cells;
- recognition and arrest within the liver sinusoid;
- cell traversal of Kupffer cells and hepatic endothelium;
- productive invasion of hepatocytes;
- switching from traversal to invasion.

What are the identities of the proteins and complexes formed on the sporozoite surface that are required for interacting with host receptors?

What is the order of interactions required for host cell invasion by sporozoites? Are proteins released from intracellular organelles in a specific schedule? How are these interactions related and different from erythrocyte invasion by *Plasmodium* merozoites, and are they conserved across other Apicomplexan parasites?

manipulate the parasite [84,85] and host [86]. Future discoveries will not only provide a molecular explanation of how sporozoites interact with host cells, but also aid the development of pre-erythrocytic vaccines that might ultimately lead to reducing and eliminating the burden of malaria.

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