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Plasmodium Sporozoite-Host Interactions From the Dermis to the Hepatocyte

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Abstract

Sporozoites are the infective stage of the malaria parasite. They are deposited in the skin by infected *Anopheles* mosquitoes and must penetrate cell barriers in the skin and liver sinusoid to reach their target cell, the hepatocyte, where they enter in a vacuole and begin development into the next life cycle stage, the exoerythrocytic form. Recent advances in our understanding of sporozoite biology in the dermal inoculation site, the role of cell traversal and the mechanism by which sporozoites productively invade hepatocytes will be highlighted in this review.

Plasmodium Sporozoites: The Same but Different

Apicomplexa is a phylum of obligate intracellular protists to which *Plasmodium* and other human pathogens such as *Toxoplasma* and *Cryptosporidium* belong. The invasive stages of these protists, called zoites, are structurally similar, possessing an apical ring of microtubules and specialized secretory organelles called micronemes and rhoptries. Work with *Plasmodium* merozoites shows that they invade cells in distinct stages, beginning with initial reversible attachment followed by irreversible attachment and the formation of a tight junction through which the parasite moves forward into the cell (reviewed in [1]). Upon entry into the host cell, the junction is sealed off and the zoite is in a parasitophorous vacuole within the cell. There is now evidence that similar to other zoites, sporozoites enter cells through the formation of a tight junction with the host cell, suggesting that the overall process is similar [2,3].

Host cell invasion by Apicomplexan zoites, including *Plasmodium* sporozoites, is an active process that requires motility [4,5]. Zoites move by gliding motility which is powered by a subpellicular actomyosin motor that is linked to the zoite surface through one or more members of the TRAP family of transmembrane proteins (reviewed in [6]). Thus, the force of the motor proteins results in the posterior movement of the TRAP-aldolase-actomyosin assembly and forward movement of the zoite.

Despite these similarities, sporozoites are different from other Apicomplexan zoites in that they are inoculated at some distance from their target cell and must make their way from the dermis to the liver in order to successfully infect the mammalian host. Thus, in contrast to many zoites, the specific biology of the sporozoite requires it to move through tissues without concomitant activation of its invasion machinery, a process that we are only just beginning to understand.

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Sporozoite inoculation into and exit from the dermis

Recent studies from several groups have clearly established that there is a skin stage of malaria infection (reviewed in [7]). Sporozoites are inoculated into the dermis by infected mosquitoes and contrary to the widely accepted notion that they rapidly leave the injection site, recent studies have shown that the majority of sporozoites that successfully reach the liver, take between 1 to 3 hours to leave [8,9]. On average, 100 sporozoites are injected by a single infected mosquito [10,11] and once in the skin, they display robust motility following what appears to be a random path rather than being targeted to blood vessels [8,12]. A proportion of these sporozoites will encounter a blood vessel, penetrate it and be carried away in the bloodstream [8,12]. The efficiency with which inoculated sporozoites exit the dermis and reach the liver has been difficult to study and awaits further investigation. What happens to sporozoites that do not go to the liver? Some are undoubtedly destroyed in the skin, some may escape destruction and remain in the skin, possibly by becoming intracellular, and approximately 20% go to the draining lymph node [8,9] where the adaptive immune response is initiated [13].

Sporozoites can migrate through cells, a process that is distinct from productive invasion and results in wounding of the traversed cell [14]. Cell traversal is required for sporozoite exit from the dermis because it enables sporozoites to penetrate cell barriers and to escape destruction by phagocytic cells in the dermis[2]. Several proteins required for this process have been identified (Table 1): SPECT (Sporozoite microneme protein essential for cell traversal;[15]), SPECT2 (also called perforin-like protein 1 or PLP1; [16,17]), CelTOS (Cell traversal protein for ookinetes and sporozoites; [18]) and PL (phospholipase; [19]). Deletion mutants of all four genes have been generated and *in vitro*, when placed directly on hepatocytes, these mutants invade and develop normally. However they exhibit little to no cell traversal activity in migration assays and exhibit a significantly decreased ability to exit the dermis *in vivo* [2,19]. The wounding of host cells by migrating sporozoites suggests that the host cell membrane is compromised during this process. How this occurs has not been elucidated but the sequence of two of the aforementioned proteins, PL and SPECT2, may provide some clues. PL has a carboxy-terminal domain with significant similarity to mammalian LCATs (lecithin:cholesterol acyl transferases) and when expressed as a recombinant protein has lipase and membrane lytic activity [19]. In addition, SPECT2 (or PLP1) contains a membrane attack complex/perforin-like domain that is similar to mammalian proteins that lyse or make holes in membranes [17]. It is clear from these studies that sporozoites have a dedicated machinery for cell traversal, further highlighting its importance to the sporozoite. Nonetheless, our understanding of precisely how sporozoites migrate through cells awaits further investigation.

In addition to cell traversal machinery, it has been recently shown that a member of the TRAP family of motor- binding proteins, called TLP (Trap-Like Protein), has a role in dermal exit [20]. Sporozoites in which this protein has been deleted display normal gliding motility on glass slides but are less successful in dermal exit. One possibility is that the force required to move through extracellular matrix is greater than that required to move on glass surfaces such that the former process may require additional motor-associated proteins to transduce sufficient force to propel the sporozoite. Alternately, it is possible that the adhesive domains of TLP interact with specific host molecules that may be required for cell traversal or crossing the endothelial cell barrier.

Crossing the Liver Sinusoid

After exiting the dermis and entering the blood circulation, sporozoites are arrested in the liver where they must traverse the sinusoidal barrier to access hepatocytes. The liver sinusoid is composed of fenestrated endothelial cells and Kupffer cells, which are resident macrophages. The endothelial cell fenestrae are too small to allow for free passage of sporozoites, therefore

sporozoites must migrate through sinusoidal cells to access the liver parenchyma on the other side. The importance of cell traversal in crossing the liver sinusoid was demonstrated when cell traversal mutants were found to have decreased infectivity *in vivo* after intravenous (i.v.) inoculation, a result that contrasts with their infectivity *in vitro* when they are placed directly on hepatocytes [15,16,18]. Importantly, these mutants regain infectivity to wild type levels, in mice pretreated with liposome-encapsulated dichloromethylene diphosponate (CL) [15,16, 18] which depletes the liver of Kupffer cells, leaving gaps in the sinusoidal barrier [21].

Switching from a Migratory to an Invasive Phenotype

How do sporozoites know they have contacted their target cell and switch from a migratory to an invasive phenotype? This is an area of some controversy. Initial studies on cell traversal raised the possibility that there is no liver-specific signal that initiates the invasion process but that after traversing a number of cells, repeated exposure to the intracellular environment activates sporozoites for invasion [22]. Thus cell traversal in and of itself is sufficient for activation for invasion. Further studies showed that the release of hepatocyte growth factor [23] or exposure to high concentrations of intracellular potassium [24] were specific events that occurred during cell traversal and led to sporozoite activation. However the generation of cell traversal mutants that cannot traverse cells yet retain full capacity to productively invade hepatocytes, raised doubts about this hypothesis, indicating that the story is likely more complex [15,16,18].

The recent finding that sporozoite residence in the dermis constitutes an important component of the malaria life cycle has led to studies of cell traversal mutants in the skin and as stated earlier, they exhibit decreased ability to exit the dermis [2]. We have found that when inoculated into the skin, sporozoites are in “migratory mode” and tend to migrate through, rather than invade, the cells they encounter. Specifically this is due to the low level of sulfation on the heparan sulfate proteoglycans (HSPGs) expressed by cells found in the dermis [25]. In contrast, when sporozoites contact hepatocytes expressing highly sulfated HSPGs they are activated via calcium dependent protein kinase 6 (CDPK6) to switch to an invasive mode [25], thus the sulfation level of HSPGs on different cell types serves as a type of “GPS” for sporozoites to know where they are and thereby control their infectivity for an organ that is far from their site of entry (Figure 1). These activated sporozoites continue to migrate through a few hepatocytes before productively invading one, thus further cell traversal may trigger other signals that render sporozoites fully competent for productive invasion. It is also possible that after initial activation by highly sulfated HPSGs, it may take some time for sporozoites to become fully competent for invasion and that further cell migration is not required. One group has proposed that the invasive capacity of the sporozoite is “masked” by the migratory phenotype so that specific signals lead not to activation for invasion but to suppression of the migratory phenotype [2]. Nonetheless, it is now clear that cell traversal is not sufficient for sporozoite activation and that there must be an initial signal that either activates or unmasks the invasive phenotype.

Invasion of Hepatocytes

Invasion by Apicomplexan zoites is an active process that requires the coordinated release of proteins from apical organelles (reviewed in [26]). Furthermore, exocytosis of apical organelles is stimulated by the mobilization of intracellular calcium and many secreted proteins contain cell-adhesive domains that function in zoite-host interactions [22,26]. Several adhesins of *Plasmodium* merozoites and *Toxoplasma* tachyzoites are proteolytically processed after their secretion onto the zoite surface (reviewed in [27]). Processing can occur in the N-terminus, C-terminus or both. N-terminal processing is thought to control exposure of adhesive domains whereas C-terminal processing is required for the release the adhesin/host cell receptor complexes from the zoite surface.

The limited amounts of sporozoite material that can be isolated has impeded the identification and functional characterization of sporozoite apical organellar proteins. To date, the best characterized proteins are CSP, the major surface protein of the sporozoite, and two microneme proteins, TRAP and AMA-1 (Table 2). CSP was originally thought to be a micronemal protein because early immuno-electron microscopy studies showed that it localized to both the plasma membrane and intracellular vesicles [28]. However, at that time there were no microneme markers to confirm the identity of these vesicles. Given that CSP is constitutively secreted onto the sporozoite surface where it forms a dense coat, it is likely that these CSP-containing vesicles are not subject to regulated exocytosis and so are not micronemes. Nonetheless, its precise intracellular localization awaits co-localization studies. The abundance of CSP on the sporozoite's surface suggests that it participates in an early step in the invasion process and indeed the specific binding of recombinant CSP to HSPGs in the liver led to the model that CSP was responsible for initial, specific attachment of sporozoites to hepatocytes but had no role in the invasion process itself (reviewed in [1]). However, our finding that proteolytic processing of CSP is associated with invasion and that E-64, a cysteine protease inhibitor, abrogates CSP processing and invasion but not attachment to hepatocytes, challenges this hypothesis [29]. CSP processing is triggered after sporozoites contact highly sulfated HSPGs [25] and leads to removal of the N-terminal third of the protein [29]. Our recent studies have shown that this event unmasks the cell-adhesive type I thrombospondin repeat (TSR) in the C-terminus of the protein which then participates in the invasion process (A. Coppi and P. Sinnis, unpublished data).

Two microneme proteins whose secretion is upregulated after hepatocyte contact are TRAP and AMA-1 [30]. Both are type I transmembrane proteins with known adhesive motifs in their extracellular domains. TRAP is the primary motor-binding protein of sporozoites [5]. Its extracellular domain contains an A-domain and a TSR motif, which have been shown to bind to highly sulfated HSPGs [31] and its cytoplasmic domain binds to aldolase which links to actin [32]. Although mutations in the extracellular domains affect invasion but not gliding motility [33], it is not clear that TRAP's role in invasion is distinct from its role in motility since altering the adhesive interactions between TRAP and its binding partners may impact on the ability of the sporozoite to move into the cell, a process which likely requires more motive force than gliding on glass.

The extracellular portion of AMA-1 has two domains with structural similarity to PAN modules which are found in a diverse array of adhesive proteins [34]. The role of AMA-1 in sporozoites has not been well studied, although studies in *Toxoplasma* tachyzoites and *Plasmodium* merozoites, suggest that it is a structural component of the moving junction [35,36]. Further studies should reveal if AMA-1 functions in sporozoites in the same manner.

After their secretion onto the sporozoite surface and possibly after binding to host cell receptors, TRAP and AMA-1 are proteolytically processed in their C-termini by one or more parasite serine proteases [30]. The protease responsible for TRAP cleavage has not yet been identified although *in vitro* data using a COS-cell assay suggests it is cleaved in the transmembrane domain by a rhomboid protease [37]. C-terminal cleavage of AMA-1 occurs in the juxtamembrane region and in the merozoite stage, the responsible protease has been identified as subtilisin-2 [30,38,39]. To date, the only protease that has been identified to have a role in sporozoite infection of hepatocytes is ROM1 (Rhomboid 1; [40]). The substrate(s) of ROM1 have not yet been identified and further studies are needed to determine the identities of the proteases that cleave TRAP and AMA-1 in sporozoites. CSP is N-terminally processed by a papain-family cysteine protease whose identity is also not yet known.

Transcription profiling of sporozoites followed by gene deletion studies have identified other proteins involved in sporozoite invasion (Table 2). P36p and P36, members of a small family

of proteins with 6 conserved cysteine residues, have been found to have a role either in sporozoite commitment to invasion or in early events after invasion [41,42]. Additionally, thrombospondin-related sporozoite protein (TRSP), a transmembrane protein with a TSR motif in its N-terminus, has also been shown to have a role in sporozoite invasion of hepatocytes [43]. Studies using immune sera have identified several other sporozoite proteins which may function in invasion, as antibodies specific for these proteins can partially block invasion (reviewed in [44]).

What are the host proteins with which these parasite proteins interact? A recent study of tight junction formation during *Toxoplasma* tachyzoite entry into cells proposed that rhoptry proteins are secreted into the host cell, insert into the host cell membrane from the inside out, and serve as binding partners for adhesins on the zoite surface [45]. Thus, the zoite may provide both the ligands and receptors it needs for invasion. This is an appealing notion and explains why after so many years of investigation, relatively few host cell receptors have been identified. In the case of the sporozoite, it is likely that highly sulfated HSPGs of hepatocytes serve as initial attachment sites (reviewed in [44]). However, downstream binding partners for the TSR of CSP, or for TRAP and AMA-1 have not been identified. Two hepatocyte proteins which clearly have a role in sporozoite invasion are the tetraspanin CD81 and the scavenger receptor SRB1 [46–48]. Tetraspanins are proteins that associate with one another and with other proteins and lipids to form membrane microdomains. To date, it has not been possible to identify a sporozoite ligand which binds to CD81, suggesting that CD81 may not itself, function as a receptor but may facilitate invasion by organizing specific proteins and lipids in the host cell membrane [46]. Since SRB1 is a major provider of cholesterol to the hepatocyte, investigators examined the organization of tetraspanin-enriched microdomains on SRB1-/- hepatocytes and found that they were significantly decreased [48]. Thus, they hypothesized that SRB1 plays a role in the formation of CD81-enriched microdomains which in turn facilitate sporozoite entry. It is possible that rhoptry proteins required for tight junction formation can more easily insert into these regions of the plasma membrane. Further studies are required to confirm that this is indeed the mechanism by which CD81 and SRB1 facilitate sporozoite invasion.

Conclusion

Recent data suggests that the process of target cell invasion by *Plasmodium* sporozoites is broadly similar to the process in their more well-studied cousins, *Toxoplasma* tachyzoites, in that it likely involves regulated exocytosis and the formation of a tight junction between host and parasite [2,3,22]. Nonetheless, the sporozoite's journey in the mammalian host dictates added layers of complexity and regulation. Its ability to migrate through nonpermissive cells [2,14] and to switch from a migratory to an invasive mode after contacting hepatic HSPGs [25] are critical to the sporozoite's ability to retain infectivity for an organ that is far from its site of entry. The precise molecular events involved in the invasion process itself remain poorly characterized, however, recent transcription and proteomic analyses specifically focusing on preerythrocytic stages of *Plasmodium* [49–51], in conjunction with new techniques for conditional mutagenesis in *Plasmodium* [52], should lead to new insights in the near future.

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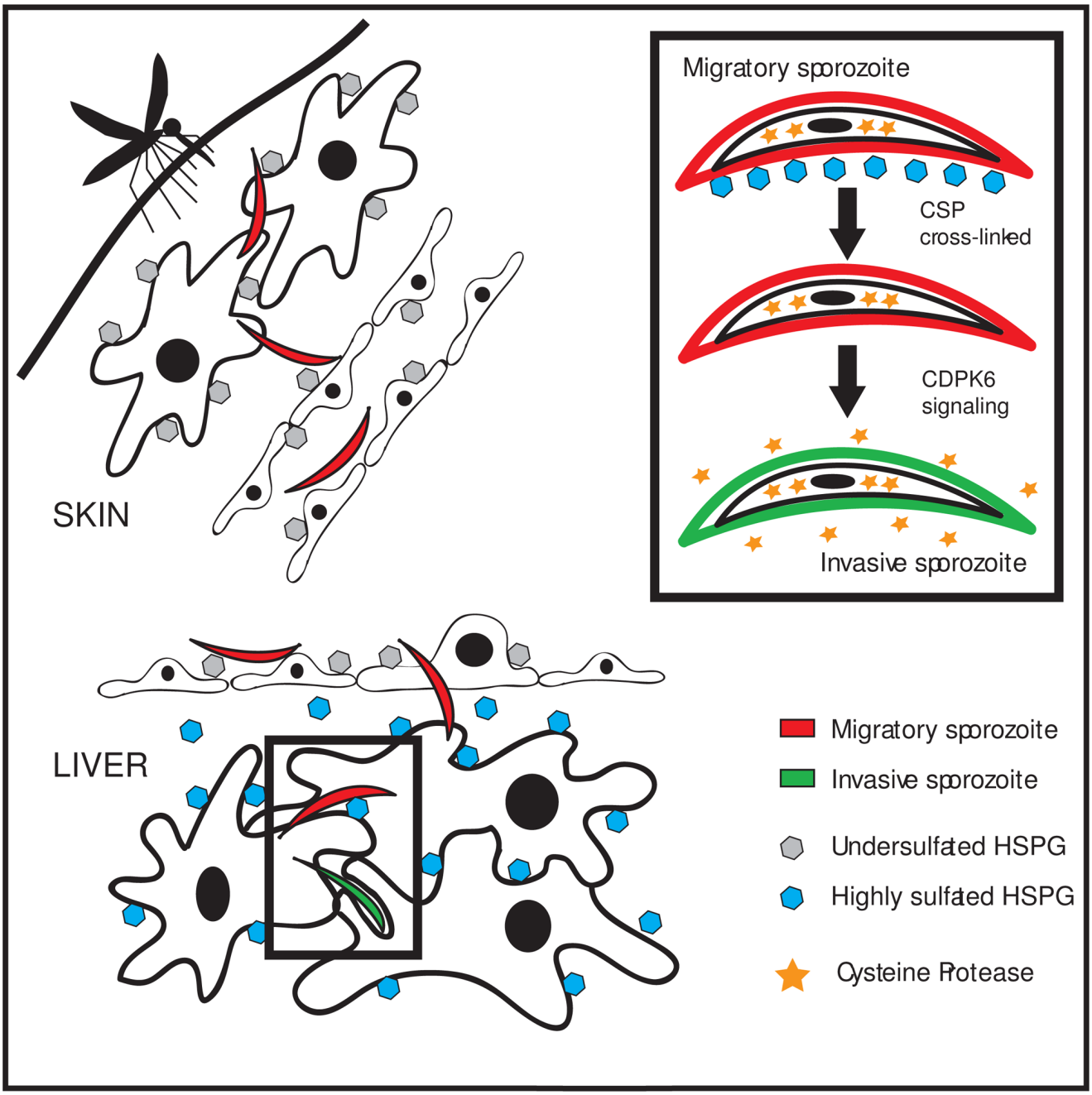

1. . Model of the Sporozoite's Journey in the Mammalian Host

Migratory sporozoites (red) are injected into the dermis by an infected mosquito, where they encounter cells expressing undersulfated HSPGs (gray hexagons) and migrate through these cells to enter the blood circulation. In the liver they cross the sinusoidal barrier and encounter the highly sulfated HSPGs found in the loose basement membrane of the liver (space of Disse) and on hepatocytes (blue hexagons) and become activated for productive invasion (green sporozoites). The inset shows some of the specific steps involved in sporozoite activation, namely crosslinking of CSP by highly sulfated HSPGs, which results in CDPK6-dependent signaling that leads to the secretion of a cysteine protease (stars) that proteolytically processes surface CSP. Reproduced with modifications and permission from [25].

Table 1

Sporozoite Proteins involved in Cell Traversal

Proteins	Method of Identification	Exit from Dermis	Exit from Sinusoid	Domains/Homologies	Reference
SPECT	Screening of EST databases	+	+	-	2 & 15
SPECT2	Screening of EST databases	+	+	MACPF domain	2,16 & 17
CelTOS	Screening of EST databases	n.k.	+	-	18
Phospholipase	Subtractive hybridization screen	+	−	LCAT	19
TLP	Microarray	+	+/−	A-domains & TSR	20

Abbreviations: MACPF, membrane-attack complex/perforin; LCAT, lecithin: cholesterol acyl transferase; TSR, thrombospondin type 1 repeat; n.k., not known.

Table 2

Sporozoite Proteins Involved in Hepatocyte Invasion

Proteins	Localization	Domains/Homologies	Reference
CSP	Surface	TSR	25 & 29
TRAP	Microneme	TSR & A-domain	5
AMA-1	Microneme	PAN modules	30 & 34
P36/P36p	Microneme	6 Cys domain	41 & 42
TRSP	unknown	TSR	43

Abbreviations: TSR, thrombospondin type 1 repeat; PAN, Plasminogen Apple Nematode