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REVIEW ARTICLE

Ali A. Sultan

Michael Heidelberger Division of Immunology, Department of Pathology, New York University School of Medicine, New York, USA

Molecular mechanisms of malaria sporozoite motility and invasion of host cells

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Correspondence to:
Michael Heidelberger Division of Immunology. Department of Pathology. New York University School of Medicine. 550 First Avenue. New York. NY 10016. USA.
Tel.: +1-212-2635346.
Fax: +1-212-2638179.
E-mail: sultaa01@mcrcr6.med.nyu.edu

Summary Malaria sporozoites have the unique capacity to invade two entirely different types of target cell in the mosquito vector and the vertebrate host during the course of the parasite's life cycle. Although little is known about the specific interaction of the sporozoite with its target cells, two sporozoite proteins, circumsporozoite (CS) and thrombospondin-related adhesive protein (TRAP), have been shown to play important roles in the invasion of both cell types. CS protein is a multifunctional protein involved in sporogony, invasion of the salivary glands, the specific arrest of sporozoites in the liver sinusoid, gliding motility of the sporozoite, and hepatocyte recognition and entry. TRAP has been shown to be critical for sporozoite infection of the mosquito salivary glands and liver cells, and is essential for sporozoite gliding motility. This review will focus on the involvement of these molecules in sporozoite motility and the invasion of host cells.

Key words Malaria · Sporozoite · Circumsporozoite (CS) · Thrombospondin-related adhesive protein (TRAP)

Introduction

Malaria parasites interact with a variety of tissue types in the vertebrate host and the mosquito vector during the course of their life cycle. When the *Anopheles* mosquito injects sporozoites into the bloodstream of the mammalian host, they rapidly invade hepatocytes in the liver, where they multiply and differentiate into merozoites. Merozoites emerging from the hepatocytes bind and invade erythrocytes within minutes of being released. Following invasion, the merozoites develop within erythrocytes. A subpopulation of merozoites differentiate into gametocytes. Male and female gametocytes ingested by the mosquito during a blood meal form gametes that mate and develop into motile ookinetes within the mosquito midgut. The ookinetes cross the midgut by invading a specific subpopulation of cells in the midgut epithelium. Underneath the basal membrane of these cells, the ookinetes develop into oocysts, in which thousands of sporozoites are formed. After a few days, the oocysts rupture, the sporozoites enter the hemolymph and selectively invade the salivary glands of the *Anopheles* mosquito. There, the sporozoites are retained until injection into the vertebrate host, completing the parasite life cycle.

Sporozoites are therefore unique among the invasive stages of *Plasmodium* in that they are invasive twice in their lifetime. In the mosquito, sporozoites invade salivary glands. In *Plasmodium* species that infect mammals, these salivary gland sporozoites rapidly invade hepatocytes, whereas those that infect avian hosts invade macrophages.

In this review, I will discuss what is known about the receptors and ligands involved in both salivary gland and hepatocyte invasion by sporozoites. In addition, I will discuss what is known of the role of motility in target cell invasion, because sporozoite invasion of host cells is more than just the sum of parasite ligands and host cell receptors. Many lines of evidence suggest that target cell invasion by Apicomplexan parasites is not a passive process in which the parasite induces its internalization by the host cell, but instead is an active process requiring the actin cytoskeleton of the parasite. An understanding of parasite motility and the way in which interactions between sporozoite ligands and host cell receptors are involved in the movement of the parasite into the cell will, therefore, lead to a better understanding of host cell invasion. The recent development of stable transformation techniques and systems for the targeted integration of exogenous DNA into malaria parasites [19, 51, 55] provides powerful tools for studying the structure

and function of *Plasmodium* molecules involved in attachment and the invasion of host cells.

Molecular interactions involved in the invasion of salivary glands by sporozoites

Plasmodium sporozoites are usually released from mature oocysts between 10 and 14 days after mosquitoes receive an infective bloodmeal. After their release from mature oocysts, sporozoites are found dispersed throughout the mosquito hemocoel, particularly in the thorax, suggesting that they are passively transported by the mosquito's open circulatory system [12]. Despite their dispersion throughout the hemocoel, adhesion of sporozoites and their major surface protein is always greater in salivary glands, suggesting a specific recognition event [12, 28]. This hypothesis is supported by recent work suggesting that antibodies that bind specifically to salivary glands inhibit sporozoite invasion [2]. In addition, Rosenberg and colleagues [33] performed a series of salivary gland transplantation experiments strongly suggesting that invasion by sporozoites is specific and receptor-mediated.

The circumsporozoite protein (CS) (Fig. 1A), the major surface protein of both oocyst [23] and salivary gland [24] sporozoites, binds to mosquito salivary glands and not to other organs exposed to the hemolymph [39]. Binding is strongest on the medial lobe and the distal portion of the lateral lobes, the portions of the glands that are preferentially invaded by sporozoites [42]. In addition, a peptide encompassing region I, a short, highly conserved sequence found in CS proteins from all primate and rodent malaria parasites, inhibits CS binding to salivary glands [39]. Of interest, the recent cloning of CS from the avian malaria parasite, *P. gallinaceum*, shows that region I and the surrounding residues are significantly different in this species [18], which is transmitted by *Aedes* mosquitoes and not by anophelines. Although further studies are necessary to determine the importance of this binding event in the life cycle of the parasite, these results raise the possibility that differences in this region of CS may, in part, determine vector competence.

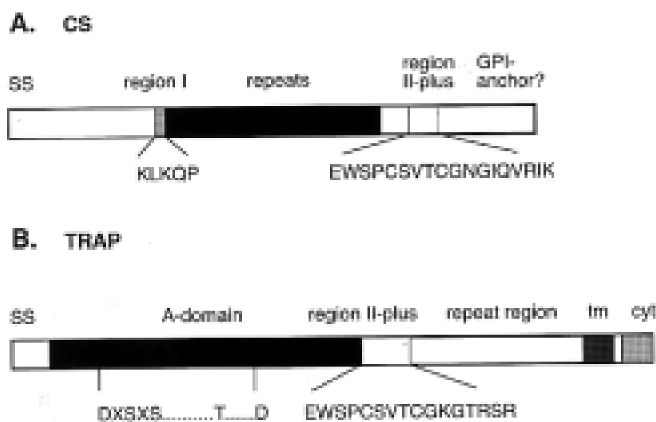

Fig. 1 (A) Schematic representation of the structure of the circumsporozoite (CS) protein showing the centrally located species-specific repeats. N-terminal and C-terminal to the repeats are two domains known as region I and region II-plus, respectively. These regions contain amino acid residues that are highly conserved in CS proteins from all species of *Plasmodium*. The amino acid sequences of these conserved regions from *Plasmodium falciparum* CS are shown. (B) Schematic representation of the thrombospondin-related adhesive protein (TRAP). Comparison of TRAP proteins from various species of *Plasmodium* shows that they all have an A-domain, approximately 200 amino acids in length. Shown is the consensus sequence of one of the most highly conserved regions of the A-domain, namely the MIDAS motif. In addition, TRAP proteins contain a region homologous to region II-plus of CS (shown is the sequence from *P. falciparum* TRAP), a transmembrane (tm) domain, and a highly conserved cytoplasmic tail (cyt). The repeat region of TRAP is an aspargine/proline-rich region, varying in length and number of repeats, with no obvious conserved sequences among the various *Plasmodium* species

In addition to CS, oocyst sporozoites possess another surface protein called the thrombospondin-related anonymous protein (TRAP) (Fig. 1B) [29, 46], also known as sporozoite surface protein 2 (SSP2) in *P. yoelii* [31, 32]. Although TRAP was originally thought to be expressed only in salivary gland sporozoites [29], recent work from our laboratory [45] has demonstrated its presence in oocyst sporozoites and that it is required for salivary gland infectivity. Furthermore, we have created TRAP null mutant sporozoites by targeted gene disruption and found that although the sporozoites were morphologically normal, they invaded salivary glands poorly, if at all. Therefore, TRAP is required for salivary gland infectivity although the mechanism involved is unknown. Other studies in our laboratory, in mammalian systems with salivary gland sporozoites, suggest that TRAP is important for target cell invasion because it is required for sporozoite gliding motility and has ligand-binding properties. Ninety percent of salivary gland sporozoites exhibit gliding motility [48], and their invasive ability is directly correlated with their ability to glide [49]. In contrast, however, only 5% of oocyst sporozoites exhibit gliding motility [48]. It is most likely that oocyst sporozoites acquire the ability to glide as they mature in the hemoceol, because a much larger proportion (about 30%) display gliding motility, and maturation may proceed asynchronously.

A recent electron microscopy study suggested that, as in other Apicomplexan parasites, target cell invasion by oocyst sporozoites is a multistep process [26]. The initial attachment of sporozoites to salivary glands involves an interaction between the parasite's cell coat and the filamentous structures of the basal lamina. Following this, the apical end of the parasite closely associates with the plasma membrane of the target cell, forming what appears to be a junction between the membranes of the target cell and the sporozoite. It is tempting to postulate that the sporozoite initially interacts with the salivary gland basal lamina via CS and that the subsequent interaction between the plasma membranes of the parasite and the target cell may involve TRAP. However, further work is necessary to determine the exact role(s) of

these proteins in salivary gland invasion.

Molecular interactions involved in the invasion of hepatocytes by sporozoites

Injection of as little as two to ten *Plasmodium* sporozoites can initiate malaria infection [14, 47]. Although it is not known precisely how many parasites are injected by a mosquito in the field, laboratory studies show that the median number of parasites injected during a blood meal is between fifteen and twenty-five [27, 33]. In addition to being efficient, sporozoite invasion of hepatocytes is rapid, occurring within minutes of intravenous injection [37]. Although hepatocytes lie beneath an endothelial cell lining, the liver is unique in that its endothelial cells have open fenestrations, allowing for direct contact between the circulatory system and hepatocytes. Estimates, however, indicate that the diameter of these fenestrations is 0.1 μm [54], about ten times smaller than the diameter of a sporozoite. One possibility is that sporozoites bind to and pass through hepatic endothelial cells to invade underlying hepatocytes [50]. It is possible that sporozoites may be arrested in the liver by sequential interactions with endothelial cell receptors similar to the way in which leukocytes roll, arrest, and extravasate at sites of inflammation. This is an attractive hypothesis because the sporozoite surface protein TRAP contains an adhesive domain called the A domain, which is also present in the leukocyte adhesion molecules LFA-1 and MAC-1 and in other proteins involved in cell-cell and cell-matrix interactions [6]. It has been shown that the binding of the A domains of the leukocyte integrins, LFA-1 and MAC-1, to the endothelial cell receptors ICAM-1, ICAM-2, and ICAM-3 [13, 16, 20, 52] mediates leukocyte arrest at sites of inflammation [41]. To test whether these molecules are important for sporozoite infectivity, we injected *P. yoelii* sporozoites into ICAM-1 and ICAM-2 knockout mice, and assessed infection of hepatocytes by sporozoites by quantitative PCR [44]. We found no difference between the knockout mice and controls, suggesting that either these receptors are not involved in sporozoite sequestration in the liver or the sporozoites can use other receptors if these are not present. Although there are currently no data supporting the transendothelial passage of sporozoites, this is an attractive hypothesis that will become testable as our knowledge of organ-specific endothelial cell markers increases.

Another possibility is that sporozoites may bind to a receptor and invade hepatocytes directly. This hypothesis is supported by the finding that CS protein, the major surface protein of the parasite, binds to hepatocyte microvilli [4]. These microvilli are the portion of the hepatocyte exposed to the circulation, and are separated from the overlying endothelial cells by a loose extracellular matrix called the space of Disse. CS protein contains a known cell-adhesive motif that is highly conserved in the CS proteins of all species of *Plasmodium* studied and is also found in the type I repeats of other cell adhesive proteins such as thrombospondin, properdin, and the neural adhesion molecules F-spondin and Unc-5. In the CS protein, this motif is called region II-plus; it is approximately 20 amino acids in length and contains an upstream tryptophan followed by the sequence CSVTCG and interspersed positively charged and hydrophobic amino acids. Recombinant CS protein lacking this region does not have binding activity, and peptides corresponding to region II-plus inhibit CS protein binding to liver sections and sporozoite invasion of HepG2 cells of the hepatoma cell line, which is permissive for sporozoite development in vitro [5, 11].

Initial studies have shown that many of the proteins containing this motif bind to sulfated glycoconjugates [5, 22, 25]. Subsequent immunoprecipitation experiments with CS protein and hepatocyte extracts demonstrated that CS protein binds to the glycosaminoglycan chains of heparin sulfate proteoglycans (HSPGs) [10]. These results were confirmed when it was shown that CS protein binding to liver sections and HepG2 cells is inhibited by treatment of the target cells with heparitinase, an enzyme that cleaves the sugar moiety from the protein backbone. Studies performed to define the structural properties of region II-plus required for binding to HSPGs demonstrated that the downstream positively-charged residues and interspersed hydrophobic amino acids were required for binding activity [40]. It is unknown whether HSPGs on the surface of hepatic microvilli extend through fenestrae into the circulation. If so, the sporozoites may be captured in the liver by the protruding HSPGs through the open fenestrae of the endothelial cells.

Salivary gland sporozoites, like those from oocysts, also express TRAP. Like CS protein, TRAP contains a region II-plus sequence homologous to that of CS protein and has been shown to have similar binding properties in vitro [22, 30]. Recombinant TRAP binds to hepatocyte microvilli in a region II-plus-dependent manner, and heparitinase treatment of liver sections abolishes TRAP binding, suggesting that TRAP also binds to HSPGS, and may be involved in sporozoite sequestration. However, the presence of the A domain suggests that it also has other functions.

In other proteins, such as the integrins, the A domain is a ligand-binding domain, binding to ligands as diverse as collagens, heparin, and the ICAMs [6]. Alignment of A-domains from various proteins reveals variable regions with short, highly conserved sequences. Recently, the crystal structure of the A-domain of complement receptor type 3 (CR3) was determined, showing that this protein contains a motif that binds divalent metal ions and is critical for ligand binding [17]. This motif, the metal ion-dependent adhesion site (MIDAS) motif, consists of a DXSXS sequence and conserved downstream threonine and aspartic acid residues. All of the TRAP molecules studied to date contain this motif, suggesting that it is critical for the function of the protein [46] (Fig. 1B). As mentioned above, a requirement for TRAP in hepatocyte invasion in vivo has been demonstrated by the

creation of TRAP null sporozoites [45]. However, the host cell receptors for the A domain of TRAP have not yet been described.

The role of motility in target cell invasion by *Plasmodium* sporozoites

In the invasive stages Apicomplexan parasites move by gliding, a substrate-dependent form of locomotion that does not involve a change in cell shape. Although it is not known how locomotion is achieved, the observation that sporozoites can translocate beads along their surface and cap cationic ferritin posteriorly has led to the hypothesis that gliding motility results from substrate-dependent capping of the surface membrane [15, 35]. According to this model, upon binding to the substrate, surface molecules spanning the plasma membrane cluster and activate a motor powered by actin-myosin interactions. As the substrate is immovable, the posterior translocation of the receptor-ligand complexes results in the forward movement of the parasite. The underlying molecular mechanism by which this is achieved is likely to be similar for all Apicomplexan parasites because, in addition to exhibiting similar patterns of motility, they also share a highly conserved structural organization that is thought to function in locomotion.

There is evidence that parasite motility is required for entry into target cells. This was suggested by early studies with sporozoites of *Plasmodium berghei* that demonstrated an association between motility and invasive capability [49]. In addition, chemical agents that disrupt microfilaments (e.g. cytochalasins), which have been shown to inhibit gliding motility, also effectively block target cell invasion [35, 43]. However, as both target cell and parasite contain actin-based cytoskeletons, it was not clear whether the inhibitory effects of cytochalasin on invasion were due to its effect on the target cell or on the parasite. This question was settled by a study performed with another Apicomplexan parasite, *Toxoplasma gondii* [8]. Using parasite and host cell mutants that were cytochalasin resistant, it was shown that, in the presence of cytochalasin, susceptible parasites cannot enter resistant cells, whereas resistant parasites can enter susceptible cells. This study definitively showed that invasion by Apicomplexan parasites is an active process dependent on the actin cytoskeleton of the parasite and confirmed the hypothesis that parasite motility is important for host cell invasion.

The mechanism of host cell entry by sporozoites, however, is still not well understood. Early electron microscopy studies using sporozoites from the species *Eimeria tenella* showed that after contact with the plasma membrane, there is a close association between the host cell plasma membrane and the anterior pole of the sporozoite [34]. The parasite then produces a parasitophorous vacuole; as it moves forward into the vacuole, the host/parasite junction moves backwards. A similar pattern of invasion has been observed for the invasive stages of *Plasmodium* [26] and *T. gondii* [21]. During invasion *T. gondii* displays on its surface a TRAP homologe called MIC-2 [53]. Like TRAP, MIC2 is a micronemal protein that contains an A domain and a sequence homologous to region II-plus. Immunolocalization studies have demonstrated a similar anterior-to-posterior movement of the protein during cell invasion [3]. These observations have led to the hypothesis that Apicomplexan parasites actively invade cells by capping the host/parasite junction posteriorly, thus moving forward into the cell [9, 15]. An exception, however, has been described for *Theileria parva*, in which parasites invade by random orientation followed by circumferential zipping between the parasite and host cell rather than by apical orientation and junction formation [36].

The finding that TRAP null sporozoites are incapable of gliding motility and are not infective for either mosquito salivary glands or mammalian hepatocytes suggests that motility is required for target cell invasion and that TRAP functions in motility and invasion by linking, either directly or indirectly, the parasite's cytoskeleton to receptors on the target cell or in the extracellular matrix [7] (Fig. 2).

Thus, TRAP and related molecules in other Apicomplexan protozoa may be central components of the motility and invasion machineries of these organisms.

Conclusion

Both oocyst and salivary gland sporozoites are released at a distance from their target organ and must therefore reach their susceptible host cells before invasion can occur. Although parasite locomotion may be involved in homing to the target organ, there is no evidence for this. Most likely, oocyst sporozoites are passively transported by the mosquito's hemolymph, and salivary gland sporozoites are carried by the circulatory system of the mammalian host. Preferential accumulation in the appropriate location is probably due to a specific recognition event that leads to arrest of the parasite. There is evidence that CS protein binding to HSPGs on hepatocytes is responsive for sporozoite arrest in the liver. The CS protein may also target sporozoites to salivary glands. Once there, however, both oocyst and salivary gland sporozoites must traverse an extracellular matrix to reach the underlying target cell. In the mosquito, the sporozoite must penetrate the basal lamina of the salivary gland before entering the secretory cell; in the mammalian host, it must traverse the space of Disse. It is likely that the crossing of these extracellular matrices requires active locomotion on the part of the sporozoite, although this has not been investigated. Both TRAP and CS protein are known to bind to components of the extracellular matrix and may be involved in this process. After the sporozoite is attached to the appropriate cell, it forms a close association with the plasma membrane of the host cell, similar to the junction

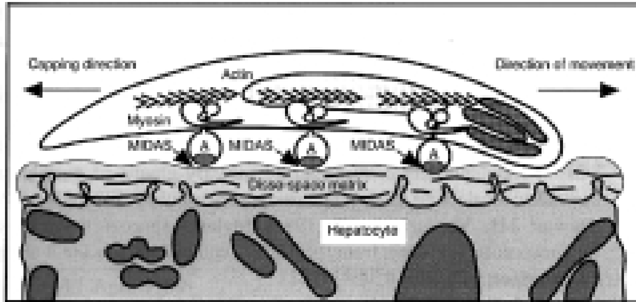

Fig. 2 Schematic model for sporozoite motility (adapted from [38]). This model postulates an interaction between the TRAP, most likely through its extracellular adhesive domains, and ligands in the Disse space and/or on the surface of the hepatocyte. This interaction, and probably others, provides the mechanical support required for sporozoite gliding and invasion. According to this model, actin filaments form the scaffold on which myosin moves forward, and along with it TRAP, which is bound to myosin directly or indirectly via its cytoplasmic tail. Backward capping of TRAP-actinomyosin complexes would result the in forward motion of the parasite

formation seen between merozoites and erythrocytes [1], and subsequently invades the target cell. We now know that TRAP is required for sporozoite infectivity in both the mosquito and the mammalian host as well as for sporozoite gliding motility. These findings, together with the localization of TRAP, during host cell entry, strongly support the hypothesis that sporozoites actively enter cells by capping the host/parasite junction posteriorly, thus moving forward into the cell. TRAP may play a central role in this process, perhaps by linking the parasite's cytoskeleton to receptors on the target cell. Although we are beginning to understand the molecular events involved in salivary gland and hepatocyte invasion, the differential infectivity of oocyst and salivary gland sporozoites in vertebrate hosts remains a mystery.

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