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Plasmodium sporozoite motility: an update

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1. ABSTRACT

Plasmodium, the causative agent of malaria, employs its own actin/myosin-based motor for forward locomotion, penetration of molecular and cellular barriers, and invasion of target cells. The sporozoite is unique amongst the extracellular *Plasmodium* developmental forms in that it has to cross considerable distances and different tissues inside the mosquito and vertebrate hosts to ultimately reach a parenchymal liver cell, the proper target cell where to transform and replicate. Throughout this dangerous journey, the parasite alternates between being passively transported by the body fluids and using its own active cellular motility to seamlessly glide through extracellular matrix and cell barriers. But irrespective of the chosen path, the sporozoite is compelled to keep on moving at a fairly fast pace to escape destruction by host defense mechanisms. Here, we highlight and discuss recent findings collected in *Plasmodium* sporozoites and related parasites that shed new light on the biological significance of apicomplexan motility and on the structure and regulation of the underlying motor machinery.

2. INTRODUCTION

Apicomplexa constitute a large phylum of parasitic protozoa, many of which are pathogenic to humans (*Plasmodium*, *Cryptosporidium*, *Toxoplasma gondii*) and/or livestock (*Eimeria*, piroplasms) (1). They are obligate intracellular organisms that actively invade host cells by developing into specialized stages, which are highly polarized and usually elongated cells. A hallmark of these pathogens is the presence of secretory organelles at the apical tip, named micronemes and rhoptries. The contents of both organelles (and that of dense granules, although not yet identified in *Plasmodium*) are critical for (i) the recognition of and attachment to the host cell, (ii) the generation of the non-phagosomal parasitophorous vacuole (PV), a cellular niche wherein the parasite will reside and replicate, and (iii) the remodeling of the host cell following invasion (2).

Apical organelles discharge a variety of type I trans-membrane surface molecules, so-called TRAP-family proteins, named after *t*hrombospondin-*r*elated *a*nonymous

*p*rotein (TRAP) (3). These proteins bear a short cytoplasmic domain, which mediates binding to the parasite cytoskeleton, and a single or multiple copies of extracellular adhesive domains of varying specificities (4, 5). Once these parasite surface ligands engage proper cellular receptors, a moving junction (MJ) is formed between the host cell membrane and the parasite (6). The MJ initially forms a cap of thickened host cell membrane covering the parasite tip and at later time points during invasion extends into a tight ring encircling the parasite and forming a distinct constriction. Several parasite proteins, e.g. *a*pical *m*embrane *a*ntigen *1* (AMA1) and *r*h*o*ptry *n*eck proteins (RON) localize to the MJ and have a key role in establishing this intimate attachment (7). Notably, recent studies in *T. gondii* tachyzoites revealed that some of them, i.e. RON2 and RON5, are targeted to the host cell membrane at the MJ where they function as binding partners for additional parasite surface adhesion molecules (8). Host cell cytoskeletal components such as filamentous actin (F-actin) and microtubules were also shown to accumulate at the site of parasite entry and to contribute to the invasion process (9, 10). Entry into the host cell proceeds as the parasite translocates the surface ligands -along with the MJ- to its posterior end while constricting and squeezing through this tight structure. This flexibility likely relies on unique features of the sub-pellicular microtubules (11). Although not yet identified, putative connection(s) between molecules that are exported from sporozoites and the host cell cytoskeleton would provide a solid anchor for pulling the parasite inside the cell. Upon entry into the host cell, the MJ is sealed off and the parasite is released into the newly formed PV.

One striking feature of many apicomplexan extracellular stages is that they move rapidly (1–5 micrometer.sec^{-1}) on solid substrates without any apparent flexing or undulation of the parasite body, nor participation of appendages such as cilia, flagella or pseudopodia. The term "gliding" is normally employed as it best describes this type of apicomplexan motility. However, it must be clearly distinguished from bacterial gliding, which involves type IV pili and/or other macromolecular structures (12). Importantly, apicomplexan gliding motility on solid substrates, capping and backward translocation of surface adhesion molecules, and host cell invasion are all intertwined phenomena driven by the same actin/myosin motor located underneath the parasite plasma membrane. Cumulative genetic, biochemical, and structural data allowed for the identification and characterization of several components of the gliding machinery. These parts of the engine were arranged into a model configuration that could explain force generation and transduction across the parasite surface (13-16). Puzzling findings regarding parasite actin dynamics (17) and the recent identification of unexpected motility modulators (18) indicate that this model is very preliminary and should be rather taken as an evolving framework to delineate future research in this field. In the present review, we highlight and discuss recent findings on apicomplexan motility, with emphasis on the *Plasmodium* sporozoite.

3. SPOROZOITE-HOST CELL INTERACTIONS

An active cellular locomotion is essential for reaching and invading host cells by most apicomplexan invasive stages. It is important to stress that *in vivo Plasmodium* sporozoites utilize their own locomotion also for extracellular migration through different tissues (19, 20) as well as for displaying a most remarkable phenotype: crossing of non-permissive biological barriers by passing through the cytoplasm of host cells.

3.1. Break on through to the other side

The so-called "cell traversal" phenotype was first described by Vanderberg and co-workers (21, 22). While carrying out video microscopy studies of *P. berghei* sporozoites/macrophage interactions they recognized an unexpected breaching of macrophages by gliding sporozoites rather than phagocytosis of the latter. The most dramatic of these events were sporozoites actively entering and exiting macrophages by piercing the plasma membrane in a "needling" fashion. The entire process was fast (< 1 min), and penetration of the macrophage was accompanied by an apparently effortless and continuous gliding of the sporozoite. This aspect raised doubts as to whether the parasite might be gliding underneath the macrophage, instead of passing through it. In some cases, however, an outward flow of cytoplasm at the site of parasite exit was noticed, unambiguously indicating that sporozoites were following an intracellular route. These findings were largely neglected for almost 25 years. Only with the advent of more sophisticated imaging and cell biology techniques, host cell traversal was revisited and corroborated as a distinctive and likely essential parasite trait (23).

In addition to the *Plasmodium* sporozoite, migration through host cells was observed for sporozoites from *T. gondii* (24) and *Eimeria papillata* (25), and for the ookinete stage of *Plasmodium* (26). Cell traversal in these parasite forms, however, is usually accompanied by significant damage to the host tissue, an aspect not observed in *Plasmodium* sporozoite transmigration. In the case of the *Plasmodium* ookinete, mosquito midgut cells typically enter apoptosis after cell traversal and are finally expelled from the epithelium by actin-based restitution mechanisms (27). The *T. gondii* tachyzoite, which does not display the cell traversal phenotype, employs a paracellular route, i.e. movement in between cells, and/or invasion of migratory leukocytes used as Trojan horses to disseminate in the vertebrate host (28).

3.2. The bipolar behavior of sporozoites

Effective host cell invasion and cell traversal activities rely on the same motor machinery and common surface molecules involved in substrate recognition and/or force transduction phenomena. However, the contrasting outcomes elicited by either phenotype pointed to the existence of a non-overlapping set of parasite molecules devoted to each process. Indeed, reverse genetic approaches carried out over the last few years allowed the identification of some of these molecules.

Disruption of the genes coding for P36p/P52 and P36, two members of a protein family that share an overall conserved structure based upon six cysteine residues (6-cys)-containing motifs (29), led to normal sporozoite formation. Knockout sporozoites, displayed normal gliding *ex vivo* on solid substrates but were deficient to replicate inside hepatocytes (30, 31), and, instead, display a 5-fold increase in cell traversal activity as compared to WT (30). These findings prompted the authors to suggest a pivotal role for P36p and P36 in triggering the sporozoite "commitment to invasion". According to this hypothesis, genetic disruption of either one or both of these proteins generates sporozoites that are impaired in turning on the invasion program, which leaves them with no other option than to continue migrating through cells until exhaustion. This notion is appealing and implicates that cell traversal is the default sporozoite behavior, which becomes masked only upon specific induction of the recessive, cell invasion phenotype. Some of the signals sensed by the parasite to kick off the invasion mode were recently described (see below) although the precise *in vivo* role for P36p and P36 as "cell invasion effectors" remains to be determined. It remains uncertain whether they are involved in host cell recognition/attachment or in early steps of hepatocyte remodeling. Regarding the latter aspect, the sporozoite-specific P36p and P36 might function in the formation/maturation of the PV, in recruiting host cell factors to the PV, or in preventing apoptosis in the infected hepatocyte (30-32). Interestingly, two other members of the 6-cys protein family, *Pfs*48/45 and *Pfs*230, are localized at the surface of *Plasmodium* male gametes and are critical for the recognition and penetration of the female gamete to form the zygote (33, 34). Irrespective of the precise molecular function, *p36p*- and other mutant sporozoites showing early cessation of liver stage development are being currently evaluated for their use as whole-organisms malaria vaccines, as they prevent the onset of blood stage infection, and thus the disease, while conferring protective immunity against subsequent challenge with WT sporozoites (35).

On the other side of the spectrum, sporozoites bearing genetic ablation of a distinct subset of surface proteins were shown to have specific deficiencies in cellular transmigration without apparent defects in gliding locomotion *in vitro* or productive invasion of hepatocytes (36-39). It is important to note that sporozoites lacking any one of these "cell traversal effectors" will invade and develop normally *in vitro* only if placed directly onto hepatocytes. Yet, *in vivo* they display severely diminished hepatocyte infectivity, since they are no longer able to breach cell barriers in the skin and the liver sinusoids. Among the "cell traversal effectors" there are two proteins that likely disturb the host cell membrane: a *p*hospho*l*ipase (PL, (37)) and a *sp*orozoite microneme protein *e*ssential for *c*ell *t*raversal-2 (SPECT-2), which contains a perforin-related domain (40). In addition, SPECT-1 (36) and other molecules, which are more likely involved in motility rather than host cell membrane disruption, such as *cel*l *t*raversal protein for *o*okinete and *s*porozoite (CelTOS, (38)) and a novel member of the TRAP family, termed *T*RAP-*l*ike *p*rotein (TLP, (39, 41, 42)), were shown to play roles in sporozoite cell traversal activity.

Cell traversal effectors were also identified in the ookinete (43). Based on the essential role of identical or functionally related molecules including CelTOS (38), the *c*ircumsporozoite- and *T*RAP-*r*elated *p*rotein (CTRP) (44, 45) and perforin domain-containing proteins (46, 47) localizing to the surface of transmigrating ookinetes it is tempting to speculate that common mechanisms might underlie the cell traversal activities of both developmental stages. Despite the apparent similarities, readers are cautioned that several data indicate that different cell traversal effectors seem to be required to break through distinct cell types or tissues. For instance, SPECT-1 and -2 are critical for breaching the sinusoidal cell layer in the liver (30) as well as for intra-dermal migration (20), whereas PL plays a role only in the latter process (37). Accordingly, the *in vivo* infectivity of PL-deficient sporozoites, but not of SPECT-1 or -2-deficient ones, can be restored to WT levels by bypassing the skin passage through intravenous injection into mice. These findings, along with the unambiguous phenotypes displayed by each individual gene knockout (both in sporozoites and ookinetes), indicate that "cell traversal effectors" are clearly not functionally redundant, and suggest that cell traversal is a much more complex parasite trait than initially thought. Unfortunately, in the absence of a clear understanding of the mechanistic basis of the cell traversal activity it is difficult to integrate these molecules into a more general scheme and/or to design a strategy aimed at identifying novel molecules involved in this phenomenon.

3.3. It takes two to tango

It is plausible that switching from cell traversal to cell invasion mode is simply the result of a time-dependent endogenous program triggered by a environmental signals, such as a temperature shift (48) or yet unknown signals sensed by the sporozoite upon being discharged into the vertebrate host. Indeed, it was shown that sporozoites can spontaneously become sessile and transform into extracellular bulb-like forms in axenic culture upon incubation at 37ºC and serum (49). However, and as typically seen in pathogen/host cell interactions, it is expected that the host cell would have at least a say in delineating the sporozoite ultimate decision: to migrate through or, alternatively, to invade and replicate inside a PV. In support of this idea, shortly after recognition of the cell traversal phenotype it was shown that this behavior leads to regulated exocytosis of secretory organelles, which in turn results in the surface display of TRAP and other molecules critical for hepatocyte recognition and invasion (50). These findings prompted the authors to put forward the hypothesis that unknown signals released from the wounded cells would activate sporozoites for effective invasion (50). In the same line, it was later shown that repeated exposure of sporozoites to certain intracellular conditions, such as high concentrations of potassium or uracyl derivatives, which activate potassium channels, can indeed enhance parasite infectivity (51, 52). The underlying mechanisms, again, likely concur with calcium mobilization and microneme discharge.

Alternatively, host cell signals were proposed to act in a paracrine way on neighboring cells instead on

sporozoites (53). In particular, it was shown that wounding of hepatocytes by sporozoite migration induces secretion of *h*epatocyte *g*rowth *f*actor (HGF), which binds to its receptor on neighboring hepatocytes and renders them more susceptible to infection (53, 54). Although potassium, uracyl and HGF directly or indirectly appear to induce a phenotype switching in the migrating sporozoite and promote its productive invasiveness, the original "invasion-activated hypothesis" (50, 53) has led to some controversy. As mentioned, multiple sporozoite mutants deficient in cell traversal activity are still fully capable of invading hepatocytes, clearly indicating that this phenomenon is dispensable for effective invasion. Current evidence actually suggests the exact opposite relationship, that is, cell traversal prevents rather than induces the cell infection pathway (20).

A recent study took up again the invasion-activated hypothesis by proposing that the sporozoite use the sulfation level of *h*eparan *s*ulfate *p*roteoglycans (HSPGs) on the surface of host cells as both a Global Positioning System and a phenotype switcher (55). According to this idea, while interacting with cells expressing under-sulfated HSPG the parasite will sense that it is still outside the liver and will continue to display the cell traversal phenotype. However, upon coming across cells covered with highly sulfated HSPG, as seen primarily on hepatocytes, it will switch to an invasion mode. The attractiveness of this hypothesis stems from a) the possibility, although at variance with previous proposals (50, 53), that sporozoite invasion does not rely on prior cell traversal activity in the liver parenchyma and b) specification of the environmental signal, HSPG, and the corresponding molecular sensor on the parasite surface, *c*ircum*s*porozoite *p*rotein (CSP).

CSP is a multitasking molecule restricted to the *Plasmodium* genus that has a key role in sporozoite development, motility, invasion and subversion of host cell mechanisms (56-58). Due to its abundance, surface localization and immunogenicity, CSP constitutes the leading candidate molecule for the development of malaria pre-erythrocytic vaccines (59). It has been long known that CSP binds to the glycosaminoglycan chains of HSPG, with the degree and type of sulfation being major determinants of this recognition (60). Coppi and co-workers demonstrated that upon engagement with highly sulfated HSPG CSP is cross-linked on the surface of the sporozoite, and that this capping effect results in a *c*alcium-*d*ependent *p*rotein *k*inase *6* (CDPK6) signaling pathway (55). This signaling pathway is apparently associated with the secretion of a, yet unknown, parasite cysteine protease that processes surface CSP and, more importantly, leads to the triggering of the invasive phenotype. Accordingly, treatment of sporozoites with general cysteine protease inhibitors, which impair CSP trimming, inhibited infectivity while causing a 5-fold increase in their cell traversal activity (55, 61). Even more compelling, incubation of sporozoites with soluble heparin, which likely precludes CSP capping, has the opposite effect (61). Together, these results strongly suggest that CSP interaction with HSPG and its subsequent trimming is one of the molecular events triggering the sporozoite invasion program. It remains to be determined what the precise consequences of CSP processing in terms of cell invasion are and whether expression, processing and/or localization of P36p and P36, the aforementioned proteins also involved in sporozoite "commitment to infection", are also affected by CSP trimming.

The hypothesis put forward by Coppi and co-workers offers a plausible mechanistic basis by which sporozoites could navigate through different mammalian tissues without unleashing their infectivity until reaching the hepatocyte. Certain loose ends remain to be clarified, though. One of these aspects is the nature of the signaling pathway triggered by CSP cleavage. Considering that CSP inserts into the outer leaflet of the sporozoite plasma membrane by a glycosylphosphatidyl inositol anchor, signal transduction across the parasite surface must involve additional CSP interacting proteins and/or lipid rafts, neither of which has been yet identified. In addition, the existence of HSPG on virtually all tissues from the vertebrate host or the mosquito host (62) may confound the traveling sporozoite. The recent description of sporozoites invading and partially replicating directly in the mammalian dermis (63) are also difficult to accommodate within this framework and corroborates the notion of a still-evolving model that can explain the intricate molecular events that happen during transformation from a fast-gliding, elongated sporozoite to a sessile, intracellular round parasite.

4. THE *PLASMODIUM* SPOROZOITE TORTUOUS JOURNEY

For the completion of its life cycle, the sporozoite must properly alternate between mutually exclusive phenotypes (migration vs. invasion) according to its location. In this section, we recapitulate the sporozoite journey focusing on this basic dichotomy: to settle and replicate or to keep on moving. However, certain aspects of the sporozoite journey cannot be easily nailed down to either phenotype. In particular, variations in the method by which the parasite migrates through cells are evident and imply additional layers of complexity and regulation.

4.1. Salivary glands: the coast in the mosquito ocean

Sporozoites bud off from the oocyst and are eventually released directly into the bathing hemolymph in a protease-driven process (64). Thereafter, they are passively transported through the entire open circulatory system of the mosquito (65). A thorough description of the process of sporogony and the multiple interactions established by the sporozoites and the salivary gland is beyond our scope, and interested readers are referred to some comprehensive reviews (66, 67).

In order to gain access to the salivary gland cavities, attached parasites first need to breach the basal lamina that covers the hemocoel-exposed side of the glands (68). During this step, sporozoites apparently strip off of their surface coat, a phenomenon best described for merozoites invading red blood cells (69). Afterwards, they

penetrate the secretory acinar cells, from the basal to the apical side, mimicking a cell traversal phenotype. However, certain central aspects substantially differ from the sporozoite migration through hepatocytes. For instance, a MJ between the invading sporozoite and the basal side of the acinar cell can be readily observed. Furthermore, intracellular parasites are initially wrapped by a PV-like structure, originating from the invagination of the acinar cell membrane (68). This PV rapidly disintegrates and free parasites accumulate in the cell cytoplasm, where host mitochondria surround them. On their way out, sporozoites do not pierce but rather "invade" the apical membrane of acinar cells with the generation of a new and transient vacuole in order to reach the interior of the salivary glands.

Once there, sporozoites re-synthesize their surface coat and either aggregate in stagnant bundles or remain actively motile within the cavity and ducts of the organ for several days (70). Imaging of sporozoite discharge through the proboscis of salivating mosquitoes indicates that sporozoite motility inside ducts favors their early ejection (70). One key aspect of the sporozoite journey inside the mosquito is the irreversible transformation from a virtually non-infective form while in oocyst to a highly infectious one inside the salivary gland. Despite their morphological likeness, the biological magnitude of this transformation, which roots in significant changes in gene expression (71, 72), is such that midgut and salivary gland sporozoites may well be considered distinct developmental forms. Indeed, both populations of sporozoites display dramatic differences at the phenotypic level when parameters such adhesiveness, surface antigen profiling, ability to elicit protective immunity in the vertebrate host and motility are considered (73, 74). What triggers this developmental program is still a mystery. But what emerges from a number of recent phenotypic studies (75-77) point to an endogenous program rather than an environmental trigger.

4.2. Under your skin

After being discharged into the host dermis, sporozoites have a limited time (up to a few h) of active motility (19, 78-81). A fraction of these sporozoites trickle out of the avascular inoculation site and eventually move into dermal blood vessels, from which they reach their target destination in hepatocytes and develop into *e*xo-*e*rythrocytic *f*orms (EEFs) (78, 79, 81). Instead of reaching a capillary, a proportion of sporozoites is flooded away by lymphatic vessels, which lead them to the proximal draining lymph node (79). To avoid lymphatic clearance, parasites are compelled to display fast extracellular motility and cell traversal activities to reach a safe capillary (Figure 1A). In addition to the obvious role in rupturing endothelial barriers, recent studies underscore the importance of active motility in ensuring sporozoite escape from skin-resident phagocytes and other immune cells recruited to the inflammation process triggered by the mosquito bite (20). Many basic aspects regarding sporozoite migration in the skin, such as directionality, a potential paracellular route or a classical transcellular route, as detected in the liver parenchyma (23), and the roles of the extracellular matrix, e.g. collagen or elastin-based structures, remain to be solved. Recent intra-vital approaches demonstrated that, at variance with *ex vivo* movements displayed by purified salivary gland sporozoites, intra-dermal motility follows what appear to be random trajectories (18, 20, 78). This erratic pattern likely rule out the influence of chemotactic signals in sporozoite recognition of the blood/lymphatic vessels and, more importantly, stresses the importance of the nature and topology of the sporozoite environment on its motility (see below).

Not all of injected sporozoites make it to the blood or lymph vessels. A proportion of them, which likely varies with many factors, such as the parasite species and degree of maturation, remain immobile in the skin. It was recently found that a fraction of these parasites might even develop in the dermis and in the immunoprivileged hair follicles (63), potentially leading to an alternative pre-erythrocytic development that is largely reminiscent of the original description of EEFs in a cultured lung fibroblast cell line (82). The finding that some sporozoites inoculated into the skin never reach the liver but remain at the injection site (and its draining lymph node) may have an impact on malaria vaccinology. Work in Balb/c mice showed that after inoculation of irradiated *P. yoelii* sporozoites into the skin, naive $CD8^+$ T cells are primed in the lymph node draining the site of inoculation by dendritic cells, and that removal of the lymph node abrogates protection (83). These antigen responses were specific for the H2-K^d-restricted immunodominant CS epitope. Although it needs to be experimentally proven, it is tempting to speculate that elicitation of protective immune responses will be enhanced by sporozoites that remain in the skin due to cell motility and/or cell traversal defects.

4.3. Home sweet home

Skin-deposited sporozoites that breach into blood vessels are rapidly floated away into the blood circulation and transported to the liver sinusoids (Figure 1B). Once there, the next challenge that sporozoites need to overcome is to cross the endothelial barrier to come in direct contact with the hepatocytes, their definitive home. As a first step toward this goal, parasites must first get off the bloodstream by firmly adhering to the sinusoidal cell layer. Mounting evidence indicates that CSP recognition of HSPG protruding into the sinusoidal lumen through endothelial fenestrations is the leading force driving this process (57). The strength of this interaction is evidenced by the fact that neither the shear force of the blood plasma nor the repeated clashes with passing blood cells are able to release attached parasites from the sinusoidal cell wall (19). Following adhesion, sporozoites start gliding either with or against the direction of the blood flow, likely scanning for a proper point of entry to the space of Disse and the underlying hepatocytes. The aforementioned endothelial fenestrations are too small to allow sporozoite passage, leaving the parasites with a single option: to go through the sinusoid endothelium. Although controversial and rather counterintuitive, multiple sets of data including the use of transgenic mice (84) and intra-vital microscopy (19) point to the resident macrophages known as Kupffer cells and not the endothelial cells as the sporozoite main gateway into the liver. It must be pointed out that passage of sporozoite

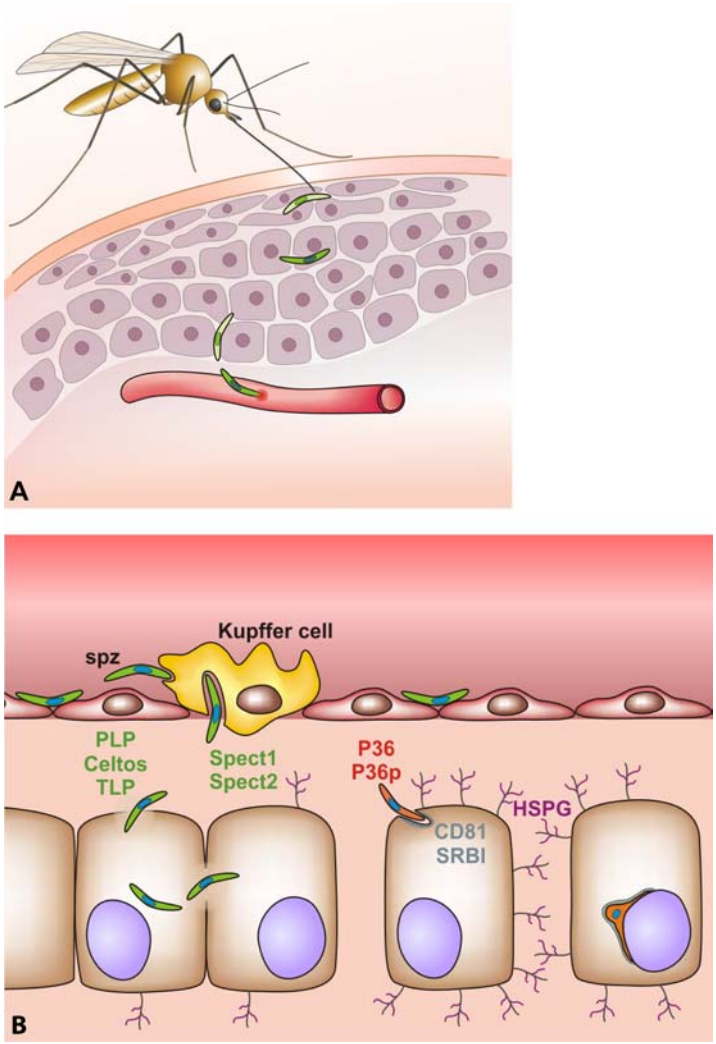

Figure 1. *Plasmodium* sporozoite transmission and targeting to the hepatocyte. A) Mature sporozoites injected into the dermis by an infected mosquito display fast gliding motility and cell traversal activities and either move between cells by a paracellular route (light green parasites) or by a transcellular route (dark green parasites) in search for a capillary. B) Exemplified for the sporozoite passage in the liver sinusoids, the motility-related functions and the parasite molecules that drive these processes are shown. Migrating and invading sporozoites are shown in green and red, respectively. Inside the liver sinusoids sporozoites adhere to the sinusoidal cells (red) and start gliding. They finally enter into the liver through Kupffer cells (yellow), apparently inside a transient vacuole. Before establishing productive invasion, sporozoites traverse through several hepatocytes (brown), by expressing specific cell traversal effectors (green). Upon contact of cells covered with HSPG, sporozoites activate invasion effector molecules (red). Cholesterol-rich micro-domains (grey), containing cluster of differentiation 81 (CD81) and scavenger receptor class B member 1 (SRBI), are critical for sporozoite invasion, perhaps indirectly by providing the proper lipid composition for the formation of the parasitophorous vacuolar membrane (grey). Upon successful invasion the sporozoite transforms adjacent to the host nucleus (blue).

through Kupffer cells proceeds at much slower speed than hepatocyte transmigration (0.1 vs 1.5 micrometer.sec^{-1}). In addition, this process involves a pause before entry and a constriction on the sporozoite body during passage (19), which morphologically resembles the formation and backward translocation of a MJ. Confocal and electron microscopy studies indicate that sporozoites are at least transiently enveloped within a vacuole while traversing the Kupffer cells (85), although it may be argued that these particular parasites were not going through but rather in the process of being phagocytosed. Altogether, and as in the case of salivary gland colonization, sporozoite migration across the inner sinusoid lining involves several features attributed to cell invasion.

Once in the liver parenchyma, sporozoites invade hepatocytes by means of yet undefined receptor(s), develop into EEFs, undergo schizogony and produce thousands of merozoites (86). Merozoites do not display gliding motility on solid substrates, but are nonetheless tailor-made to

invade erythrocytes by a similar active and multi-step invasion process described for the sporozoite (87). But before establishing productive infection, sporozoites traverse through several hepatocytes, as if searching for the proper nurse cell where to settle. Current evidence indicates that crossing hepatocytes is detrimental for the sporozoites *in vivo* as it causes the release of several host cell factors such as NF-kappa beta, which can alert the immune system (88). Going back to the Global Positioning System hypothesis (55) and putting the pieces together, it may be speculated that migration through hepatocytes occurs because of a certain delay between engaging HSPG, or additional signals that alert the sporozoite to a "liver environment", and unfolding the molecular and cellular events triggering the invasion program. This apparently disadvantageous behavior may have been selected for in the *Plasmodium*-mammal co-evolution as a means to modulate, or even limit, the parasite population of a malaria infection.

5. GLIDING MOTILITY

5.1. The road

Ex vivo assays with freshly isolated parasites moving on an artificial substrate, i.e. albumin- or extracellular matrix-coated glass slides, or cultured cells have greatly boosted our cellular and molecular understanding of Apicomplexa movement and adhesion. After the initial description of gliding locomotion by *T. gondii* tachyzoites (89) and *P. berghei* sporozoites (90), a detailed analysis established a reproducible assay to monitor and quantify sporozoite gliding locomotion, a continuous, often circular or helical, forward motility that occurs without obvious changes in cell shape (21). The technique is very straightforward: it only requires freshly collected parasites, which are suspended in a quite simple medium containing albumin or serum. Observational studies using related apicomplexan parasites, such as *Eimeria*, the causative agent of coccidosis in poultry and a wide range of wild animals, *Gregarina*, a diverse group of invertebrate parasites, and *Cryptosporidium*, causing acute intestinal infections, contributed to a unifying view of apicomplexan cellular motility (91-94).

In spite of its simplicity and continuous use throughout the years, two basic features of the *ex vivo* motility model remain largely unknown: a) the nature of the substrate anchoring the parasites to the glass and b) the identity of the receptor(s) on the parasite surface and the exact mechanism by which albumin or undefined serum component(s) trigger parasite motility. The latter point will be discussed in more detail in the next sections, as it is related to the mechanisms by which the parasite senses and transduces the environmental cues. As for the nature of the anchoring substrate, it has been hypothesized that, in the absence of external sources, sporozoites gliding on glass slides need to provide their own substrate (56). In this line, a recent study on *T. gondii* tachyzoites invasion suggests that proteins secreted from the rhoptries insert into the host cell membrane at the MJ, and serve as binding partners for other parasite surface adhesion molecules (8, 95). An analogous mechanism in which parasite surface molecules are inserted into the copious vesicles and/or lipid-rich structures secreted from the anterior tip of sporozoites and function as grip for its subsequent gliding might be envisaged to explain extracellular gliding locomotion.

A major drawback of *ex vivo* assays is that they constitute oversimplified models that do not take into account external, and major, determinants of *in vivo* parasite motility such as substrate topology, endothelial barriers, phagocytic cells and shearing forces. Other supports such as flexible gels (which might be further enhanced by adding cells) were recently developed to partially overcome these limitations. Indeed, and in contrast to glass slides where sporozoites maintain a fixed crescent shape and move mostly in circles or spirals with occasional twists and turns, it was shown that parasite migration in matrigel is characterized by high parasite flexibility and more erratic trajectories involving frequent changes in direction (Figure 2). These "corkscrew" patterns are much more alike to trajectories followed by sporozoites in the dermis and the liver parenchyma, as revealed by intra-vital microscopy techniques (18-20).

It is important to note that the geometry (2D or 3D) and the nature of the extracellular environment regulate the pattern of sporozoite motility by imposing different topological constraints but also by affecting the way in which parasite force is transduced. At the size scale of sporozoites, Brownian motion rules over gravity, i.e. the weight of the parasite itself is not sufficient to maintain surface contact. Hence, migrating parasites are strictly dependent on adhesion receptors to remain anchored to the substrate and to resist strong shear forces *in vitro* (96) and *in vivo*, particularly towards the end of the journey, in the lumen of the sinusoid (19). Using 2D substrates, it was recently shown that adhesion receptors, at least the operating ones, are not evenly spread over the entire cell surface but concentrated on discrete and dynamic membrane "patches", termed adhesion sites (96). These previously unrecognized features of sporozoite motility were evidenced by quantitative imaging techniques, so-called reflection-interference and traction-force microscopy. In case of 3D substrates, where the parasite is tightly surrounded by fibrils or cell surfaces, substrate anchoring likely becomes more and more dispensable and transmission of traction forces alone might be sufficient to move the cell, as was elegantly demonstrated in migrating leukocytes (97). Although the mechanical basis underlying this phenomenon is not fully understood, it seems that only the sheer force of polymerizing/depolymerizing actin filaments -and thereby pushing perpendicularly against external surfaces- might be sufficient to allow locomotion in the absence of force coupling.

Recent studies using sporozoites deficient in a small heat shock protein, HSP20, further emphasize the major impact of the extracellular environment on sporozoite motility (18). HSP20-deficient sporozoites display aberrant speed and trajectories when gliding on glass slides, which correlate with the presence of a single large adhesion site spanning almost the entire parasite length. Although the molecular basis is not yet understood, it is plausible that mutant sporozoites cannot regulate the

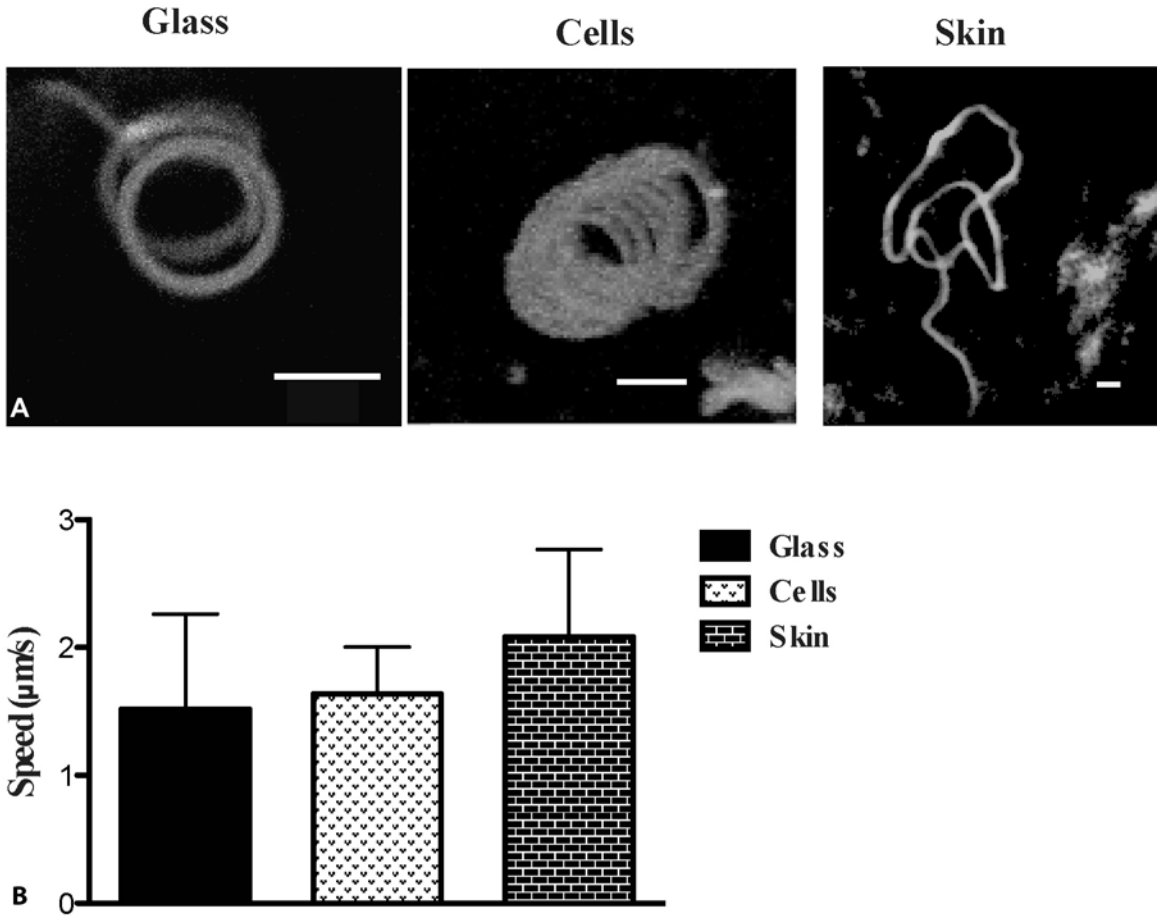

Figure 2. *Plasmodium* sporozoite motility. A) Z-projections of serial time lapse micrographs of *P. berghei* sporozoites on albumin-coated glass slides (left), cultured fibroblasts (center), and inside the mouse skin (right). Note that sporozoites describe circular trajectories on glass and fibroblasts, and elongated at random trajectories *in vivo*. Scale bars: 5 micrometers. Time series: 50" (glass), 175" (cells), 140" (skin). B) Average speed of *P. berghei* sporozoites on different substrates. Shown are mean values (± S.D.) of sporozoites gliding on a glass surface (n=20); on cultured fibroblasts (n=5), and within the mouse dermis (n=5).

cytoskeleton dynamics required to generate traction forces for attachment and detachment cycles properly. Interestingly, *hsp20*- sporozoites follow similar trajectories, albeit at reduced speed, as WT parasites when deposited in the skin, indicating that aberrant *in vitro* directionality observed can be compensated for when sporozoites move in natural 3D environments. Moreover, the fact that the overall speed reduction of *hsp20*- sporozoites is similar when gliding on glass slides or in the dermis suggests that transmission of traction forces and not adhesion phenomena drive parasite motility in natural, complex 3D organs.

5.2. The motor

Observational studies led to the proposal that the actin/myosin-based molecular motor is located on the parasite periphery, more specifically in the narrow sub-pellicular space between the *i*nner *m*embrane *c*omplex (IMC) and the plasma membrane. This localization integrates well into the original model, i.e. that capping and rearward translocation of trans-membrane adhesion proteins by stationery motor particles would thus translate into forward movement of the parasite (94). The overall premise turned out to be correct, and molecular studies aimed at identifying the main components of the gliding machinery as well as their sorting mechanisms, molecular topology and interactions over the last few years have essentially supported this distinct mode of cellular motility.

The current status of knowledge places the motor machinery, which includes the myosin heavy and light chains and their anchoring proteins, tethered to IMC (98). The IMC is composed of flattened cisternae derived from the endomembrane system that extends beneath the entire surface of the parasite, except at its very apex, and is supported on their cytoplasmic side by microtubules and a network of ill-defined filaments (99-102). The myosin driving parasite motility is *myo*sin *A* (MyoA) (103-105), which belongs to an unconventional class of "neckless" myosins (class XIV) that is unique to Apicomplexa (103, 106). Myo A is tethered to the IMC *via* its calmodulin-like, myosin-light chain (termed MTIP in *Plasmodium* sporozoites and MLC-1 in *T. gondii* tachyzoites) and at least two gliding-associated proteins, GAP45 and GAP50

(107-109). GAP45 is highly conserved among apicomplexans (although restricted to the phylum) with myristoylation, palmitoylation and phosphorylation sites (110, 111). A recent elegant study demonstrated that this protein interacts not only with the IMC but also with the plasma membrane, and could thus serve as a molecular ruler anchoring both structures while preventing the collapse of the cortical space (111).

In *T. gondii*, the integral protein GAP50 recognizes soluble pre-complex motor particles assembled in the cytosol (containing MyoA–MLC-1–GAP45) and anchors them in the cholesterol-rich membrane of the IMC (110). Direct or indirect connection of GAP50 to the network of microtubules underneath the IMC would likely provide a much better support for this protein and hence the whole motor, but the required connections, if they do exist, remain elusive. Recently, it was shown that GAP40, a polytopic protein of the IMC highly conserved across the phylum apicomplexa, co-immunoprecipitates with the MyoA-MLC-1-GAP45-GAP50 complex, strongly suggesting it is another component of the motor complex (111). Additional polytopic proteins residing in the IMC and termed glideosome-*a*ssociated *p*rotein with *m*ultiple-membrane-spans (GAPMs) were identified in *P. falciparum* late blood stages by proteomic analysis of detergent-resistant membranes (112). GAPMs were shown to form very stable high molecular weight complexes, which led to the idea they could be components of the missing link anchoring the IMC to alveolins and/or other components of the underlying cytoskeleton. Further studies are required to ascertain the *in vivo* role, if any, for these molecules in invasion.

As part of the motor complex, MyoA moves along short and dynamic F-actin, which in turn interacts with the cytoplasmic tail of TRAP-family proteins, likely *via* fructose 1,6-phosphate aldolase tetramers (113, 114). "Moonlighting" activities of aldolase, i.e. supplementary functions unrelated to catalysis, have been demonstrated in other systems (115) and are consistent with its F-actin-binding properties and the huge aldolase concentration in eukaryotic cells, largely exceeding the glycolytic requirements. Recent structural studies provided detailed information regarding protein-protein interactions within the motor (116-119), and provided clear support to the role of aldolase as an intermediate scaffold. However, a combined biochemical and molecular genetics approach in *T. gondii* demonstrated that energy production rather than adhesin-cytoskeleton bridging, is the essential role of aldolase in parasite gliding locomotion. Although the aldolase-binding sites for fructose 1,6-phosphate and the TRAP tail partially overlap, mutations in a series of positive residues lining the catalytic pocket of aldolase demonstrated that both catalytic and scaffolding activities of aldolase could be dissociated. Conditional complementation with these aldolase mutants permitted the distinction between enzymatic activity and binding to the tail of the MIC2, a surface adhesion molecule belonging to the TRAP family, in living tachyzoites. Overall, these experiments indicate that MIC2 linkage to aldolase, and thus to the cytoskeleton, plays a role when traction is required, i.e. during host cell invasion, but is less crucial or even dispensable during *ex vivo* gliding (120). In addition of further stressing the importance of the environment on parasite motility, these findings revives the search for additional molecular link(s) that transmit rearward movement of F-actin to the TRAP invasins and, hence, the outer world.

Once the entire complex reaches the posterior tip of the parasite it needs to be disengaged, a process that involves protease-mediated shedding of TRAP proteins (121). Upon release, these molecules are deposited along with other parasite proteins and lipids on a continuous trail left behind moving parasites, a signature feature of gliding locomotion in apicomplexan parasites.

5.3. The regulators

The structural and functional basis of the machinery core propelling Apicomplexan parasites appears to be largely unraveled. In spite of this outstanding progress, most of the aspects underlying the overall regulation of this molecular motor are still unknown. Regulation of parasite motility can occur at several levels including actin dynamics, trafficking and processing of motor components, and energy supply.

5.3.1. Actin dynamics

According to the present model, the mechanical force driving parasite motility seems largely generated by the immobilized myosin (Figure 3). However, the susceptibility of apicomplexa to actin polymerizing/depolymerizing drugs, e.g. cytochalasin D, jasplakinolide, and latrunculin A, indicate that filament formation is a rate-limiting step in *Plasmodium* motility (96, 122-125). Furthermore, actin dynamics also seems to directly regulate parasite adhesion to substrates before gliding (74). Most apicomplexa express a single actin gene, which codes for one of the most divergent actins known so far. Indeed, this molecule exhibits unusual dynamic properties: while *in vitro* studies have shown that it can be rapidly polymerized into microfilaments at 3-4 fold lower critical concentration than mammalian muscle actin, it is maintained largely in a globular state within the parasite under physiological conditions (124, 126, 127). These findings, along with the paradoxically limited repertoire of classical actin-binding proteins compared with that of other eukaryotes (17, 125) point to the presence of additional unidentified proteins orchestrating actin dynamics in apicomplexa.

In general, actin regulators in eukaryotic cells are involved in three principal processes: actin monomer (G-actin) tread-milling, nucleation/organization of the actin filament network and stabilization of F-actin (128). Three distinct binding proteins involved in G-actin tread-milling have been described in apicomplexa thus far: profilin, cofilin/*a*ctin-*d*epolymerizing *f*actor *1* (ADF1), and the small *c*yclase-*a*ssociated *p*rotein (C-CAP). Profilin binds to and activates actin monomers, thus facilitating their incorporation at the barbed-end of pre-existing filaments (128). Conditional disruption of the profilin gene in *T. gondii* showed it is essential for gliding motility, host cell

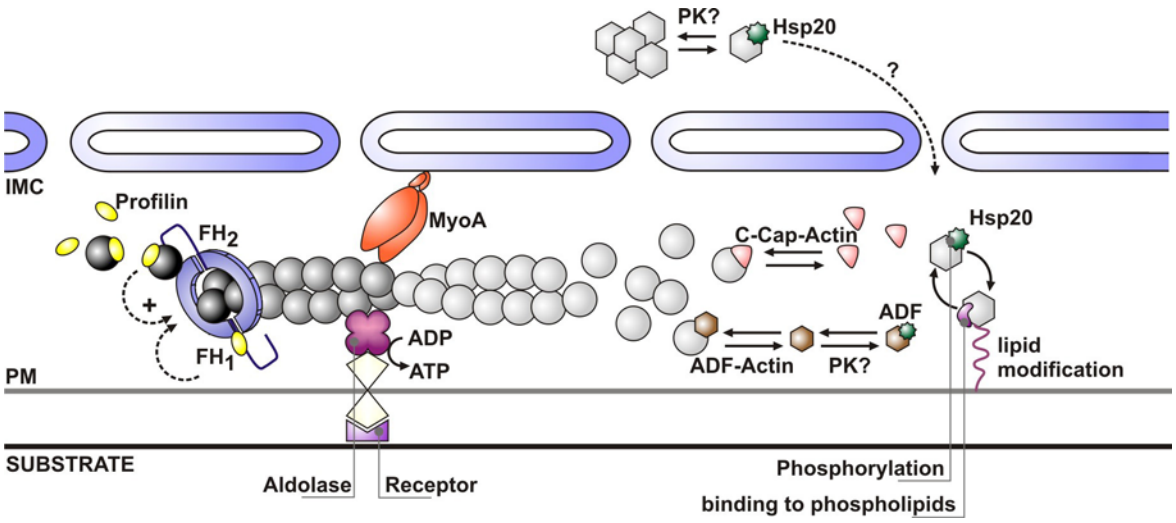

Figure 3. Hypothetical model of microfilament regulation during *Plasmodium* sporozoite gliding locomotion. The *Plasmodium* motor machinery is placed between the inner membrane complex (IMC), and the parasite plasma membrane (PM). Immobilized myosin (MyoA) walks along short actin filaments, which are indirectly attached to extracellular receptors on the target cell *via* aldolase tetramers (purple) and trans-membrane receptors of the TRAP family (white). Length of actin filaments is regulated by G-actin sequestering proteins: ADF1 (brown), profilin (yellow), and C-CAP (pink). Formins (FH1 and FH2) might participate in actin polymerization at the barbed ends recruiting G-actin (circles)/profilin complexes. Actin binding proteins may be regulated by posttranslational modifications, e.g. phosphorylation (exemplified for ADF1). HSP20 can modulate speed and directionality, perhaps by directly interfering with myosin-actin interactions, by changing the membrane fluidity or by competing with ADF-phosphorylation. Positioning of regulators around the motor machinery may be modulated by lipid modification, binding to phospholipids and/or phosphorylation (all exemplified for HSP20). The stimuli and receptors that initiate motility are largely unknown, but the signaling process involves calcium release, protein kinases (PK) activation and phosphorylation cascades that could ultimately influence in the activities of actin-binding proteins.

invasion and egress, and virulence in a mouse model (129). Intriguingly, profilin, like bacterial flagellins (130), is recognized by the host innate immune system as a ligand. Profilin recognition by the *t*oll-*l*ike *r*eceptor *11* (TLR11), the only TLR that is apparently restricted to rodents and absent in humans, elicits Myd88-dependent defensive immune responses (131). How *T. gondii*, and not *Plasmodium*, profilin gets in contact with TLR11 remains unclear, particularly since recent evidence suggests that TLR11 is localized in the cell interior (132). In *Plasmodium*, profilin seems to be expressed in most of motile stages of *Plasmodium* and has a vital role in blood stages (133).

Cofilins/ADFs, on the other hand, are involved in assembly/disassembly of actin filaments by promoting pointed-end depolymerization and subsequent sequestration of actin monomers (128). *Plasmodium* expresses two ADFs, with ADF1 being the major isoform in motile stages. ADF1 and its orthologue in *T. gondii* display strong actin monomer-sequestering activity but show a weak filament disassembly capacity, likely because they lack key F-actin binding motifs (126, 134). Notably, these molecules promote the conversion of ADP-actin monomers into ATP-actin monomers, a function normally assisted by profilins. Finally, small C-CAP orthologs were identified in Apicomplexa (17, 125). *Cryptosporidium* and *Plasmodium* express functionally interchangeable C-CAP molecules displaying a single G-actin binding domain and unique structural features (135). Apicomplexan C-CAPs show the strongest actin monomer-sequestering activity of all G-actin binding proteins, which has a net detrimental effect on F-actin formation. In *Plasmodium,* this protein has a vital role during oocyst development, thus precluding generation and potential analysis of mutant sporozoites (135). Overall, the combined effect of these three abundant proteins (profilin, ADF-1 and C-CAP) is consistent with actin being maintained largely (>98%) in a globular state in apicomplexan parasites. Unfortunately, current technical limitations preclude the functional analysis of these molecules in *Plasmodium* sporozoites.

In the absence of conserved gene orthologues for ARP 2/3 key components and its additional nucleation promoting regulators such as ENA/WAVE/WASP, the most appealing actin nucleator candidates in Apicomplexa are formins. This idea is consistent with the current model of gliding in Apicomplexa, which places a myosin molecule fixed to the IMC walking along short homopolar actin filaments, although the visible constriction of sporozoites at the MJ during invasion supposes the generation of cortical tension, which could involve F-actin reorganization underneath of parasite plasma membrane. It was initially shown that both apicomplexan formins (FRM1 and FRM2) nucleate chicken actin polymerization *in vitro*, and that one of them (FRM1) localized to the MJ established by *Plasmodium* merozoites and *T. gondii* tachyzoites, thus suggesting a role in host cell invasion (136). Further studies using conditional gene disruption and expression of dominant mutants in *T. gondii* allowed the

functional dissection of both formins in this organism (137). According to this analysis, TgFRM1 is preferentially positioned at the plasma membrane, where fast nucleation can occur in close proximity to the complex formed between F-actin and the aldolase-MIC2 tail complex. The filaments likely elongate over only a short distance, with TgFRM2 potentially serving to stabilize and control their size close to the IMC. Interestingly, TgFRM1 and TgFRM2 deficiency preferentially affected helical and circular gliding, respectively, illustrating distinct contributions of the two formins in parasite motility. In spite of above mentioned findings in merozoites (136), there is no experimental evidence yet for a role of formins in *Plasmodium* sporozoite motility or invasion.

A few additional regulators of actin dynamics were identified in apicomplexa. In *P. falciparum*, one coronin-like protein was shown to co-sediment with F-actin *in vitro* (138). In mammalian cells, coronins are proposed to interact with ARP2/3 and F-actin to prevent cofilin-mediated disassembly of newly formed filaments at the leading edge (139). Another molecule displaying both G-actin-sequestering and filament capping activities has been described in *T. gondii* (140). This protein, termed toxofilin, localizes to the rhoptries (141) and seems to be secreted during invasion (142), although its functions remain uncertain. More recently, a direct role in motility was demonstrated for an actin *c*apping *p*rotein *beta* subunit, CP beta, and a dynein light chain in *Plasmodium* (75, 143). In the case of CP beta, loss-of-function parasites display partial and complete gliding defects in ookinetes and sporozoites, respectively (75). Therefore, CP beta is the first actin regulator that exerts a vital role in *Plasmodium* sporozoite motility. Novel candidates for cytoskeleton regulators in Apicomplexa might be small heat shock proteins. In *T. gondii*, HSP20 co-localize with the motor complex at the outer surface of the IMC (144) and genetic ablation of this molecule in *Plasmodium* leads to abnormal turnover of sporozoite-substrate adhesion sites (18). In good agreement, HSP20 was proposed to directly or indirectly modulate actin dynamics in mammalian cells, in addition to its role as molecular chaperone (145).

5.3.2. Trafficking and processing of motor components

One critical aspect underlying sporozoite motility is that every component of the motor machinery needs to be properly positioned at the right time and place to interact with their partners. As mentioned, calcium-mediated exocytosis, triggered upon binding of albumin, underlies discharge of apical organelles and surface translocation of adhesion molecules (146). Additional cAMP and cGMP signaling pathways are also involved in microneme secretion (147). At least three CDPKs seem to be involved in signaling pathways leading to motility and invasion (148). CDPK3 is required for gliding motility and invasion of midgut cells by *Plasmodium* ookinetes (149, 150). Drug inhibition of CDPK1 supported its implication in *T. gondii* tachyzoites motility and invasion (151). A CDPK1 ortholog is expressed in the asexual blood stages of *Plasmodium* and malaria parasites treated with CDPK1 specific inhibitors causes developmental arrest at the schizont stage (152). Finally, CDPK6 triggers sporozoite egress out of midgut oocysts and the subsequent invasion program in *P. berghei* sporozoites (55, 153), providing a molecular association for a shared machinery for parasite egress and invasion. While the major role appears to be the triggering of organelle apical discharge, these or other kinases might also initiate the gliding motor upon activation. The best characterized substrate for CDPK1 is GP45, whose phosphorylation was shown to be critical for the final step of glideosome assembly in *T. gondii* (110). Other appealing candidates for phosphorylation-induced activation include ADFs, toxofilin and small heat shock proteins. HSP20 displays an intriguing pattern of polarization in *P. berghei* motile sporozoites, indicating that it is indeed transiently recruited to the parasite pellicle during gliding (18).

The proper positioning of the motor complex to the parasite pellicle is mediated by post-translational lipid modifications, in particular myristoylation and palmitoylation (111). Notably, most of apicomplexan CDPKs contain acylation consensus sequences, which might regulate their intracellular localization and transient association to the IMC or plasma membrane. These modifications could also cause the proper positioning of most other regulatory proteins in the pellicle, such as ADFs and HSP20. Moreover, it was recently shown that enzymes participating in palmitoyl recycling might modulate motility and/or invasion (154). Interestingly, aldolase and other glycolytic enzymes relocalize from the parasite cytoplasm to the pellicle following *T. gondii* egress from host cells (155). The molecular basis underlying this cortical accumulation remains to be ascertained but it is apparently not mediated by covalent modifications, such as palmitoylation. It is tempting to speculate that the biological role, if any, of this physical association of glycolytic enzymes is to improve the overall efficiency of local ATP production by channeling reaction intermediates between individual enzymes.

An alternative mechanism to recruit proteins to the parasite pellicle (either the plasma membrane or the IMC) may involve regulated interaction with phospholipids, especially inositol phosphates. These phospholipids influence activities of many actin binding proteins in other systems, as shown for profilins and actin nucleators (139), and can thus be involved in directionality of cell movement and/or chemotaxis. Two proteins involved in motility are prime candidates for potential interactions with phospholipids in Apicomplexa, *Pf*profilin and *Tg*HSP20 (133, 144). Recent analyses showed that certain glideosome-related proteins, such as GAP50, MyoA and Myo B/C as well as key components of the MJ (RON2 and AMA1), are *N*-glycosylated in *T. gondii* tachyzoites (156), whereas aldolase, FRM2 and actin are oxidatively modified in intraerythrocytic stages of *Plasmodium* (157). The impact of these and other post-translational modification in the intracellular trafficking/function of these molecules remains to be determined. It is highly likely that additional, and, perhaps, even parasite-specific regulatory mechanisms will be identified that together initiate, modulate, and disassemble the motor complex multiple times throughout the sporozoite journey.

6. PERSPECTIVE

Decades of research have revealed different aspects of the complex process of sporozoite motility. In spite of this wealth of information, several open and fundamental questions still remain to be answered: How is actin dynamics orchestrated in this parasite? What are the precise molecular mechanisms underlying sporozoite migration through cells? How many variations in this phenotype do exist and how are they regulated? What are the environmental cues and downstream signaling pathways mediating cell traversal-to-invasion switching? In the absence of *in vivo* techniques for conditional mutagenesis in *Plasmodium*, more powerful image capturing and analyzing techniques, advanced and high through-put "omics" assays and comprehensive libraries of small molecules will be instrumental to address these issues.

Over 125 years after the discovery of its causative agent, malaria remains one of the most devastating infectious diseases in the world, with an estimated toll of 200 million new cases resulting in ~1 millions deaths, primarily infants and young children in SubSaharan Africa, every year. Malaria control efforts suffer from widespread resistance to anti-parasitic drugs and insecticides, underpinning the urgent need for novel intervention strategies. In this sense, the molecular motor emerges as an attractive source of potential new ways of intervention (152, 158-160). The essential role of this machinery, its overall conservation across the phylum and the presence of unique proteins that are absent from the host and display a number of unusual features including unconventional myosin and actin molecules indicate that a better understanding of its *in vivo* functioning and regulation may eventually lead to new chemotherapeutic approaches for the treatment and causal prophylaxis of malaria and other diseases caused by apicomplexan parasites.

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