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Invited Review

Apicomplexan cytoskeleton and motors: Key regulators in morphogenesis, cell division, transport and motility

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ABSTRACT

Protozoan parasites of the phylum Apicomplexa undergo a lytic cycle whereby a single zoite produced by the previous cycle has to encounter a host cell, invade it, multiply to differentiate into a new zoite generation and escape to resume a new cycle. At every step of this lytic cycle, the cytoskeleton and/or the gliding motility apparatus play a crucial role and recent results have elucidated aspects of these processes, especially in terms of the molecular characterization and interaction of the increasing number of partners involved, and the signalling mechanisms implicated. The present review aims to summarize the most recent findings in the field.

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1. Introduction

This review describes the most recent progress made in understanding the contribution of cytoskeletal elements and protein motors in governing the conserved mechanisms behind the Apicomplexa lytic cycle events (Fig. 1). This phylum includes many parasites, and while some aspects of their life cycle, such as the machinery that leads to gliding motility and the process of invasion, have been extensively dissected and demonstrated to be shared among the members of the group, others, such as cell division and morphogenesis, or host cell egress, are poorly understood. The most recent data suggests that both conserved and specific mechanisms are involved in these processes.

2. Apicomplexan cytoskeleton – taking advantage of the best of two worlds: stability and flexibility

2.1. The apical complex and cytoskeleton

The invasive stages of the apicomplexan life cycles are named zoites. These highly polarized cells attach apically to host cells and invade them by building a unique membranous structure named the parasitophorous vacuole (PV). This distinct form of invasion, as well as migration, replication, invasion and egress (exit from host cells) takes advantage of the high flexibility of the cytoskeleton that while, on one side, assures maintenance of the parasite's cell shape and structural integrity, on the other side, enables adjustment of this same shape during migration and host cell invasion. Consequently, while some characteristics of the apicomplexan cytoskeleton are conserved with those of other eukaryotes, others are unusual and specific to these organisms.

Apicomplexan parasites are delimited by the pellicle (Fig. 2), a tri-bilayer structure, comprising the plasma membrane and two tightly associated membranes formed by endoplasmic reticulum (ER)-derived flattened vesicles named the inner membrane complex (IMC). The IMC extends throughout the body of the parasite and provides support for the gliding machinery, which drives motility. Closely associated to the parasite pellicle is the subpellicular network, which acts as the parasite's skeleton and is constituted by the intermediate filament-like TgIMC1 (Mann and Beckers, 2001). In contrast, an updated annotation of the *TgIMC2* gene reveals that it codes for a phosphatase-like protein that carries a signal peptide and is the resident protein of the IMC (Frenal and Soldati, unpublished data). One of the IMC-associated proteins, photosensitized INA-labeled protein 1 (PhIL1), is thought to be responsible for the cytoskeleton-pellicle association (Gilk et al., 2006). Underneath the subpellicular network, at the apical tip, is

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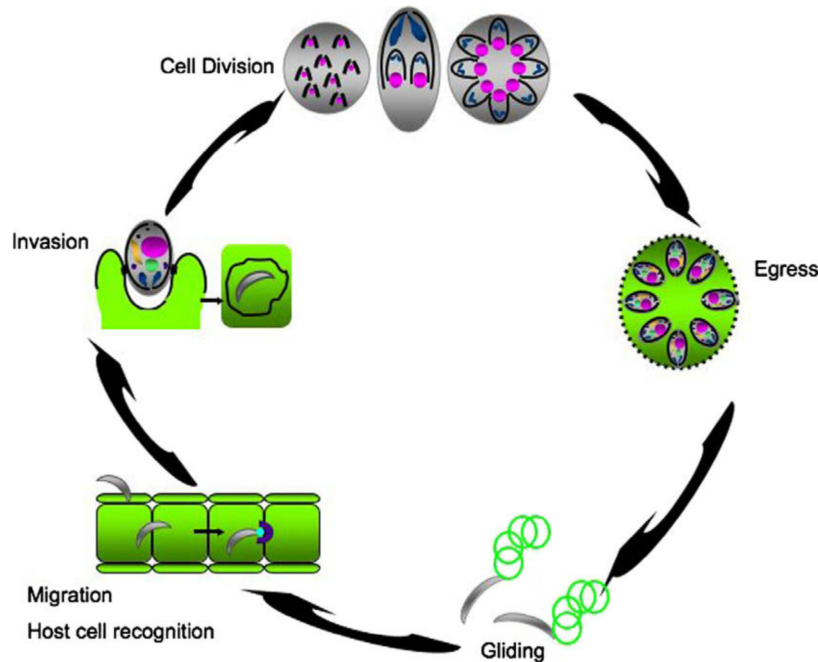

Fig. 1. Apicomplexan life cycle illustrating all the lytic events: parasite division inside the invaded host cell, egress of the new daughter cells from the host cell, gliding motility, migration inside the host and recognition of the right target cell, and invasion of the new target host cell (clockwise).

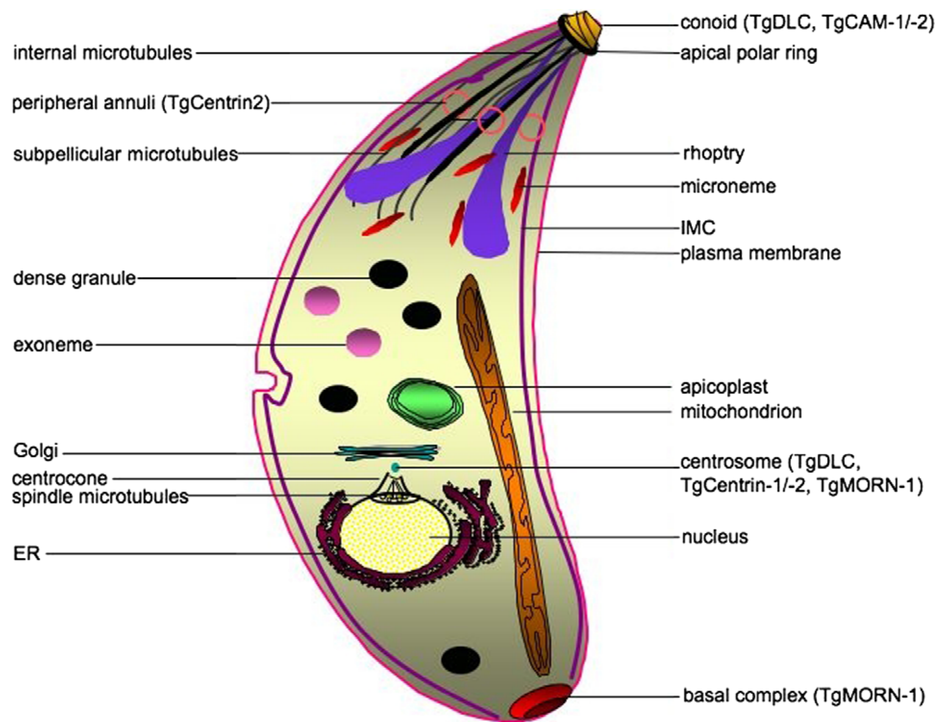

Fig. 2. Scheme of a "model" apicomplexan parasite. Shown are the cytoskeleton elements (microtubules and centrocone), the apical complex (micronemes, rhoptries, conoid and apical polar ring), the pellicle (inner membrane complex (IMC) and plasma membrane), the secretory organelles (exonemes, dense granules, micronemes and rhoptries), the non-secretory intracellular organelles (mitochondrion, apicoplast, nucleus, endoplasmic reticulum (ER) and Golgi) and the basal complex. Note that not all members of the phylum contain the full repertoire shown in the figure.

the apical complex, after which the phylum is named, and the basal complex is localized at the opposite end (Gubbels et al., 2006).

The apical complex is an exclusive structure of this group of parasites but its composition can vary depending on the members of the phylum; whilst the specialized secretory organelles, micronemes, rhoptries and dense granules, as well as the apical polar ring, are present in all Apicomplexa, the full repertoire, which includes the conoid, is only present in a set of parasites named coc-

cidians. The apical polar ring and the conoid are both elements of the cytoskeleton but while the first one is the microtubule organizing center (MTOC) of the subpellicular microtubules, the conoid is organized into a hollow cylinder composed of a polymer of alpha and beta tubulins assembled into a new type of protofilament sheets (Hu et al., 2002) and can move up and down through the apical polar ring and protrude apically at the time of cell invasion in a calcium-dependent fashion (Mondragon and Frixione, 1996; Monteiro et al., 2001). Three proteins likely to be involved in this motility are dynein light chain (TgDLC), which could be part of the motor, and calcium-binding proteins 1 and 2 (TgCAM-1 and -2), which may regulate this kind of motion (Mondragon and Frixione, 1996; Hu et al., 2006).

As mentioned above, the subpellicular microtubules, which are important for shape, apical polarity and organelle trafficking, are organized from the apical polar ring, but two other sets of microtubules exist in these parasites. One set is found in the mitotic spindle, where it coordinates chromosome segregation and originates from MTOCs organized by centrioles in Coccidia (Dubremetz, 1973), or by rudimentary spindle pole bodies in other Apicomplexa such as *Plasmodium* (Schrevel et al., 1977), and the other set is localized in the conoid. These different microtubules are uniquely specialized, in a phenomenon reflective of the apicomplexan parasites' lifestyle. For instance, it was recently found that the subpellicular microtubules of *Plasmodium berghei* sporozoites (Cyrklaff et al., 2007) are maintained in a state of "suspended depolymerization" by an as yet unidentified molecule that allows them to bend far beyond what is allowed by regular microtubules undergoing treadmilling, an ability that is especially important during the transmigration and invasion processes.

The number and organization of the microtubules can also differ between parasite species and even life cycle stages. *Cryptosporidium parvum*, for instance, was shown not to have subpellicular microtubules but longitudinal ridges that might perform a similar function (Matsubayashi et al., 2008), and *Besnoitia besnoiti* (Cortes et al., 2006), another coccidian parasite, was shown to change shape and modify its surface when invading host cells, due to re-arrangements of its cytoskeleton, despite having the same subpellicular microtubule organization as the other Coccidia.

2.2. Building zoites: different ways of dividing; same mechanism?

Different Apicomplexa, and even different life cycle stages of the same species, adopt distinct strategies to ensure the completion of their replicative cycle. Most intracellular stages are not infectious and therefore cell division has to be precisely timed in order to ensure that the new daughter zoites are fully formed and prepared to invade at the time of host cell egress. The usual rule is schizogony, where several rounds of DNA synthesis and nuclear division occur prior to zoite genesis and cytokinesis, but in some cases parasites replicate via endodyogeny (a variant form of schizogony where DNA replication is immediately followed by nuclear division and cytokinesis), which leads to the production of only two new daughter cells per replication cycle. Regulation of this process seems to involve cell cycle checkpoints similar to those of other eukaryotes reviewed in Gubbels et al. (2008b), as a forward genetic screen of temperature-sensitive *Toxoplasma gondii* cell cycle mutants led to the identification of multiple proteins, several of which were shown to be orthologues of known cell cycle factors (Gubbels et al., 2008a). These master switches were recently suggested to be up/down-regulated according to each parasite-specific program, i.e. parasites that execute several rounds of DNA synthesis before cytokinesis (i.e. schizogony) would down-regulate proteins involved in the checkpoint at the end of DNA replication (Striepen et al., 2007). Apicomplexan mitosis is known, however, to also involve unique aspects. In *T. gondii*, for example, the S phase is bipartite (Radke et al., 2001) and in Coccidia, the mitotic spindle undergoes a complex cycle (Dubremetz, 1973), developing first extranuclearly, then becoming intranuclear and, at last,

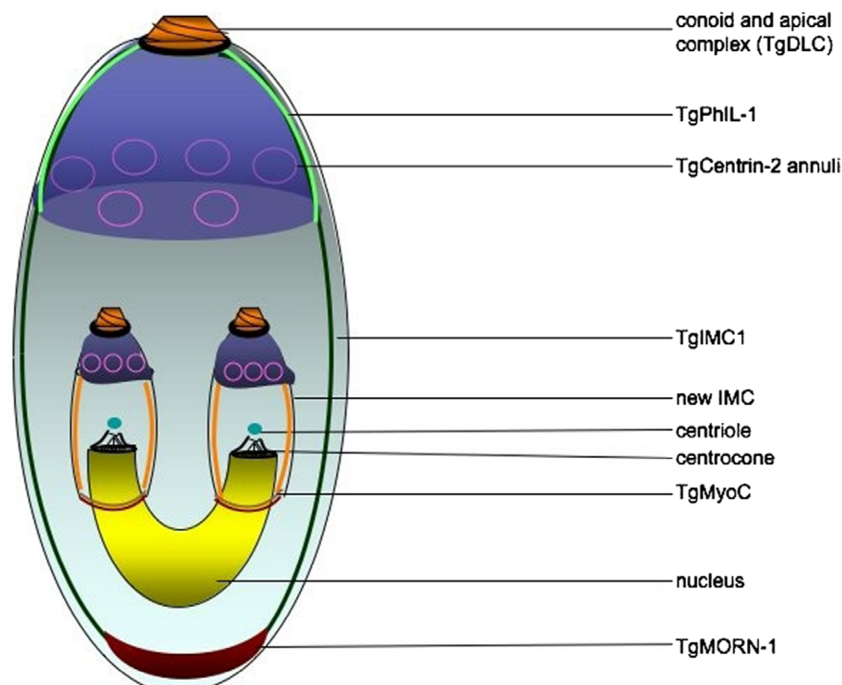

Fig. 3. Scheme of a mother parasite undergoing division by endodyogeny. Two identical new daughter cells are produced, completely protected within the mother cell until the end of the process. IMC, inner membrane complex.

turning into a pair of centrocones, derived from both the nuclear envelope and the spindle poles, and characterized by the presence of the membrane occupation and recognition nexus motif containing protein TgMORN1 (Gubbels et al., 2006).

Multiple studies performed during the past 40 years have suggested that there is no fundamental distinction between the various modes of reproduction, apart from the number of nuclear divisions preceding zoite genesis. In all cases, the morphogenesis of apicomplexan zoites has been described as being coordinated with mitosis. The mitotic poles were clearly shown to be the primary organizing centers of both the mitotic spindle and the apical cytoskeleton of the nascent zoites. The pattern of differentiation has been described in many Apicomplexa and it was found to be conserved ((Dubremetz, 1975). What has been revealed more recently are the molecular features of the structures previously described. These findings essentially concern the process of endodyogeny of *T. gondii* tachyzoites, thus we will only report on this process (Figs. 3 and 4).

The first sign of cell division is the migration of the centrioles to the basal pole of the nucleus and their replication. These replicated centrioles sandwich the spindle and the poles of the spindle give rise to the centrocones, on which the kinetochores of replicated chromosomes are attached. The primary structures which develop next to these centrioles are the two apical polar rings, from which the subpellicular microtubules extend, covered with the early IMC. The outer edges of the IMCs appear as ring structures decorated with TgMORN1 and are the precursors of the daughter basal ring complexes, remaining at the basal ends of the daughter cytoskeletons as the new cells grow (Hu, 2008). Two new conoids are constituted within the apical polar ring close to the TgMORN1 rings (Hu et al., 2006) and the growing subpellicular microtubules provide the scaffold for the construction of the new daughter IMC (Hu et al., 2002) and subpellicular network (Mann et al., 2002). An actin-like protein (TgALP-1) (Gordon et al., 2008) appears to be connected to the formation of this new IMC, either by escorting ER vesicles or by scaffolding membranes. The newly formed IMCs continue to elongate and progressively wrap the mitotic poles of the dividing nucleus together with the apical organelles (Nishi et al., 2008). Concomitantly, the new basal complexes gain polarity, upon recruitment of the centriolar marker, TgCentrin2, and the dynein light chain, TgDLC, and move away from the apical complexes in a process thought to be microtubule-dependent (Gubbels et al., 2006). The new parasites continue to expand and fill the mother until they bud out, acquiring in the process their plasma membrane. At this time there is complete closure of the basal complex in a process possibly driven by TgCentrin2 (Hu, 2008). This kind of replication ensures, that the new parasites are produced and protected within an infectious mother parasite.

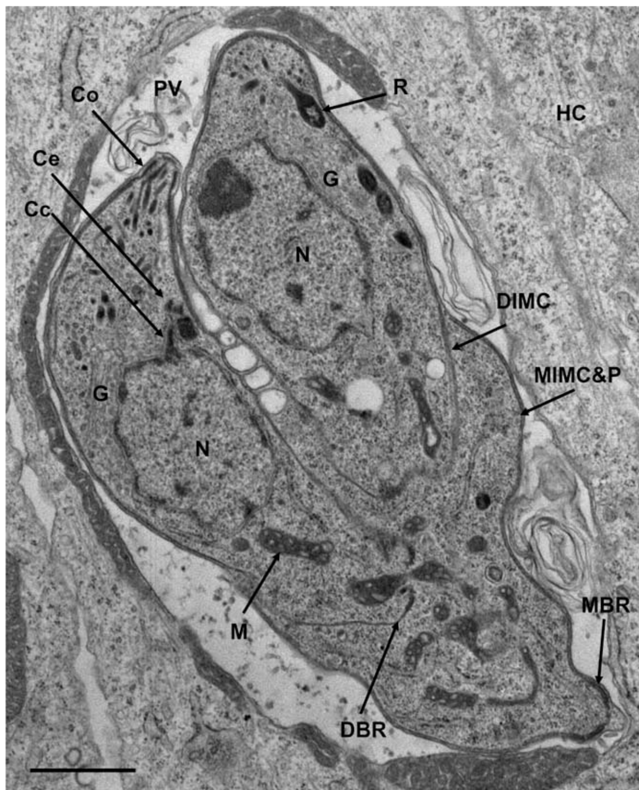

Fig. 4. Late stage of *Toxoplasma gondii* endodyogeny showing several of the cytoskeletal molecules involved in the process; Cc, centrocone; Ce, centriole; Co, conoid; DBR, daughter basal ring; DIMC, daughter inner membrane complex; G, golgi; HC, host cell; M, mitochondrion; N, nucleus; MBR, mother basal ring; MIMC&P, mother inner membrane complex and plasmalemma; PV, parasitophorous vacuole; R, rhoptry. Bar = 1μm.

3. Host cell egress: signalling and mechanism

The intracellular growth of Apicomplexa eventually causes lethal lysis of the host cells in a mechanism termed egress, which results in the exit of infective parasites from their PVs. This peculiar process occurs after a highly variable number of parasite division cycles, the general rule being that a fixed number of mitosis occurs at each schizogonic stage of the cycle. Such a mechanism ensures that the daughter zoites are completely differentiated before the host cell is damaged by parasite development. In *T. gondii*, host cell lysis is a less critical issue since endodyogenic division allows production of infective parasites at the end of every round of replication.

In *T. gondii*, egress is an active process relying on the parasites' ability to sense that their host cell is dying or dead. Treatment with agents, such as the Ca^{2+} ionophore (A23187), which leads to an increase in the intra-parasitic level of calcium, is a potent artificial inducer of parasite egress (Endo et al., 1982) as early as 2 h p.i. (Caldas et al., 2007). During this process the micronemes discharge their contents, the conoid extends and the parasites become motile.

Changes in host cell membrane permeability and host cell ionic homeostasis seem to be involved but the exact origin and nature of such a signal remains elusive. It is known that potassium (K^+) signalling is involved as decreasing the potassium concentration leads to premature egress (Black et al., 2000; Moudy et al., 2001) and the same phenomenon occurs following treatment with the K^+ ionophore nigericin (Fruth and Arrizabalaga, 2007). This mechanism is thought to involve a yet unidentified parasite sensor that detects a decrease in the concentration of K^+ inside the host cell and PV. The ability of the parasite to sense changes in the environment might also allow a prompt exit from compromised cells such as those targeted by the immune defence system.

In such a model, molecules described to be crucial for Ca^{2+} signalling, such as phospholipase C, calmodulin and a Ca^{2+}-dependent calmodulin domain protein kinase have been proposed to be required for egress and invasion (Moudy et al., 2001; Caldas et al., 2007). A more recent study revealed that *T. gondii* tachyzoites can produce the hormone abscisic acid that induces the production of cyclic ADP ribose (cADPR) and which in turn stimulates calcium-dependent protein secretion and leads to egress. The critical role played by this hormone is moreover supported by the fact that

the same effect is obtained with the addition of exogenous abscisic acid and that the selective disruption of its synthesis by the inhibitor fluridone leads to a delay in egress and prompts parasite differentiation into bradyzoites (Nagamune et al., 2008). The notion that egress depends on the parasite actin-dependent motility was recently challenged by the finding that treatment with actin-disrupting drugs does not delay parasite egress. A new model for egress was then proposed in which the disruption of host cell actin would lead to internal pressure and mechanical rupture of the host cell membrane, which in turn would activate parasite motion due to the loss of ions from the host cell (Lavine and Arrizabalaga, 2008).

In the case of *Plasmodium falciparum*, the egress of merozoites from red blood cells (RBCs) is very tightly regulated and involves the timely breakdown of the PV membrane followed by vesiculation of the RBC membrane (Glushakova et al., 2005). A new class of secretory organelles named exonemes have recently been identified and shown to control egress via release of the serine protease PfSUB1 (Yeoh et al., 2007). Indeed, PfSUB1 is discharged from exonemes into the PV space before host cell rupture and induce a proteolytic maturation of the vacuolar marker PfSERA5. This last hypothetical protease is known to be essential for the efficient release of parasites from host RBCs (Delplace et al., 1988; Yeoh et al., 2007; Arastu-Kapur et al., 2008). Similar findings have been reported for sporozoite release from mosquito midgut oocysts, which is completely prevented by the disruption of another SERA family member, SERA8 (ECP1), (Aly and Matuschewski, 2005).

4. Gliding machinery in Apicomplexa: the motor that drives infection

Migration across biological barriers and active penetration of host cells and egress rely on the parasite's ability to glide. Gliding motility is critically dependent on actin polymerization and is powered by a myosin motor (MyoA) ubiquitoulsy conserved across the phylum (Wetzel et al., 2005; Baum et al., 2006a,b; Jones et al., 2006; Schuler and Matuschewski, 2006). *Toxoplasma gondii* MyoA (TgMyoA) was originally shown to belong to a motor complex including the myosin light chain (TgMLC1) (Herm-Gotz et al., 2002) that is firmly anchored in the plane of the IMC by the integral membrane glycoprotein GAP50 and the lipid modified GAP45 (Johnson et al., 2007). This organization extends to *Plasmodium*, where the orthologues of PfMTIP, PfGAP45 and PfGAP50 have been identified in *P. falciparum* merozoites (Baum et al., 2006b; Jones et al., 2006), and to all other members of the phylum.

It has been established that aldolase offers a bridge between the actomyosin system and the host receptor-parasite ligand complexes (Jewett and Sibley, 2003; Bosch et al., 2006). This glycolitic enzyme is unexpectedly able to bind to both the C-terminal domain of an adhesin (TgMIC2 in *Toxoplasma* and TRAP, MTRAP and TLP in *Plasmodium*) and the parasite's actin filaments (Buscaglia et al., 2003; Jewett and Sibley, 2003; Baum et al., 2006b; Heiss et al., 2008). This interaction involving aldolase, and potentially other proteins, is important for parasite survival (Starnes et al., 2006). In such a model, motility is presumably generated by the posterior translocation of F-actin-aldolase bound to the adhesin proteins driven by the myosin tracks firmly anchored and immobilized in the IMC (Johnson et al., 2007).

Despite a clear role of F-actin dynamics in gliding, formal demonstration of the presence of F-actin in Apicomplexa has been difficult due to the short size and inherent instability of these filaments. Indeed, apicomplexan actin exhibits unusual properties. The majority of actin molecules are maintained in a globular state (Schmitz et al., 2005; Baum et al., 2006a; Sahoo et al., 2006; Schuler and Matuschewski, 2006) that can be rapidly polymerized into microfilaments at a concentration three to fourfold lower than mammalian muscle actin, in a process dependent on the presence of salt, magnesium and ATP (Sahoo et al., 2006). This allows a rapid treadmilling process that facilitates directional migration, the fast regeneration of new actin subunits for future rounds of assembly, and avoids unwanted locomotion (Baum et al., 2006a; Sahoo et al., 2006; Schuler and Matuschewski, 2006).

Both these actin dynamics and microfilament turnover are suggested to arise from a sophisticated interaction with a vast array of actin binding proteins (Schmitz et al., 2005). However, apicomplexan genomes contain relatively few conventional actin-binding proteins. Among this limited repertoire are actin depolymerizing factor (ADF1) (Schuler et al., 2005), capping protein alpha and beta (Gordon and Sibley, 2005), profilin (Plattner et al., 2008), toxofilin (cofilin) (Poupel et al., 2000) and coronin (Tardieux et al., 1998; Figueroa et al., 2004). Unexpectedly, Apicomplexa lack a canonical actin regulator Arp2/3 complex, which is otherwise widespread among eukaryotes where it drives actin assembly by nucleating filaments from the pointed end. Instead, the Apicomplexa possess formins (Gordon and Sibley, 2005; Baum et al., 2006a) that are known, along with profilin, to drive actin polymerization in a mechanism alternative to that of the Arps (Higgs and Peterson, 2005). In *T. gondii*, profilin was recently shown to play a vital role in parasite motility and invasion in a process conserved across the phylum, since the *Plasmodium* profilin fully complements a *T. gondii* profilin knockout strain. Furthermore, purified recombinant profilins from three different Apicomplexa are able to control actin polymerization (Plattner et al., 2008). Apicomplexan genomes also encode two or more large formins that feature a typical forming homology domain 2 (FH2) and a recent study highlighted *Plasmodium* formin 1 (PfFRM1) as a potential effector in actin nucleation during invasion, based on its localization at the moving junction and its ability to act as a potent actin nucleator of chicken actin in vitro (Baum et al., 2008).

5. Migration and host cell recognition: how to get there and sense where you are

5.1. Migration: getting there

Plasmodium sporozoites are only able to invade a restricted set of cell-types and have to endure a long journey in order to reach their final destination, making migration undeniably fundamental for the establishment of a malaria infection. Progress in investigating this phenomenon has vastly benefited from the sophisticated improvements in bioimaging (Amino et al., 2005, 2007; Frevert et al., 2005; Tarun et al., 2006; Thiberge et al., 2007), and this review will only focus on migration of the *Plasmodium* sporozoites from the site of injection to the liver. It is now known that once deposited in the skin, sporozoites do not leave immediately but remain at their site of inoculation for 1–3 h after the mosquito bite (Yamauchi et al., 2007) before entering blood or lymph vessels (Amino et al., 2006). If invasion of a blood vessel occurs, the sporozoites are carried in the bloodstream and readily reach the liver. Once in the liver sinusoids, the next barrier that sporozoites need to overcome is the endothelial barrier. It has been suggested that to access hepatocytes, sporozoites pass through the resident liver macrophages (Kupffer cells) (Baer et al., 2007). At this point the parasite circumsporozoite protein (CSP) binds to the liver surface LRP-1 and proteoglycans and prevents activation of the respiratory burst, hence contributing to

parasite survival (Usynin et al., 2007). As previously shown for *Eimeria* sp. interacting with cells in vitro (Roberts et al., 1971), when encountering the hepatic cells the sporozoites do not immediately establish infection but first traverse several hepatocytes (Mota et al., 2001). It was initially hypothesized that this would occur so that the host cells would be activated and become more receptive to infection (Carrolo et al., 2003), but this theory was revised when transgenic parasites lacking sporozoite microneme proteins essential for cell traversal (SPECT-1 and -2/PPLP1) were shown to be unable to migrate through host cells but nonetheless were able to productively invade hepatocytes (Ishino et al., 2004, 2005b). Given that SPECT mutant parasites are less infective than wild type ones in vivo using the rodent malaria model, it appears that host cell traversal is not essential but might help the parasite to encounter the optimal host cells (Amino et al., 2008). In contrast, crossing hepatocytes also causes the release of several host cell factors such as NF-κB, which can alert the immune system and limit the extent of malaria infection in the liver (Torgler et al., 2008).

In addition to SPECT-1 and SPECT-2 (Ishino et al., 2004, 2005b), two new members of the TRAP family TLP (Moreira et al., 2008) and TRSP (Labaied et al., 2007), CelTOS (Kariu et al., 2006) and a phospholipase PbPL (Bhanot et al., 2005) have been reported to be involved in cell traversal, however little is known about their mechanistic contribution to the process.

Several homologues of these proteins have been identified in ookinetes. MAOP is a SPECT-2 homologue (Kadota et al., 2004) and CelTOS is expressed at both sporozoite and ookinete stages (Kariu et al., 2006) suggesting that a common mechanism might explain membrane rupture of the hepatocyte and of mosquito midgut cells.

New steps were also made towards elucidation of the signals that induce this type of migration. Calcium-dependent protein kinase 3 (PbCDPK-3) was shown to regulate ookinete invasion of the midgut wall (Ishino et al., 2006) and PbCDPK-6 was demonstrated to be involved in the switch between migration and invasion (Coppi et al., 2007). This calcium signalling is conserved across the Apicomplexa phylum, as *T. gondii* CDPK-1 (TgCDPK-1) appears to regulate motility and host cell invasion (Kieschnick et al., 2001). Potassium signalling also contributes to migration given that exposure of parasites to high concentrations of potassium leads to a decrease in migration (Kumar et al., 2007), and activation of a potassium channel stimulates apical exocytosis, which causes a decrease in cell traversal (Ono et al., 2008).

5.2. Host cell recognition: how to know it is time to stop migrating and start invading

Apicomplexa exhibit very diverse preferences in terms of host and host cell-type specificities with some parasites being able to invade a wide repertoire of host cells while others are extremely restricted.

A generally common feature of host cell recognition seems to involve the binding to sialic acids on receptors at the surface of host cells. In *Plasmodium*, the erythrocyte surface protein 175 (EBA-175) binds to the heavily sialyated receptor glycophorin A of RBCs (Tolia et al., 2005). A similar type of interaction might govern *Babesia bovis* binding to erythrocytes as the presence of a sialyated receptor similar to glycophorin A was shown to contribute to host cell invasion (Takabatake et al., 2007). Recognition of sialic acid was also previously reported to be critical for *T. gondii* invasion (Monteiro et al., 1998). More recently, the adhesive domain called microneme adhesive repeat (MAR), present on TgMIC1, was demonstrated to bind selectively to sialic acid (Blumenschein et al., 2007) however the nature of the receptor(s) on the host cell surface awaits further investigations.

A set of new data concerning recognition of liver cells by *Plasmodium* sporozoites revealed that the level of sulfation of surface hepatocyte glycoproteins named HSPGs serves as a local positioning system (Coppi et al., 2007). The sporozoites seem to be "activated" for invasion when they contact the highly sulfated HSPGs of the hepatocytes due the induction of the proteolytic processing of CSP that occurs just prior to invasion (Coppi et al., 2005). Pbs36p and Pbs36 are two members of the 6-cys domain-containing proteins family that participate in this process of commitment for invasion as disruption of these genes leads to continuous traversal of hepatocytes and failure to find suitable host cells (Ishino et al., 2005a).

Despite intense studies on the malaria liver stage, a receptor for sporozoites on hepatocytes has yet to be identified. CD81, a tetraspanin family member, is involved in the permissiveness of hepatocytes to infection but this role seems to be indirect since CD81 appears to act as a modulator of an unidentified sporozoite protein receptor (Silvie et al., 2003a,b; 2006; Yalaoui et al., 2008). New clues regarding the answer to this question may be provided by the analysis of the *belr1* locus of chromosome 17, which encodes several host cell genes involved in susceptibility of mice to a liver infection (Goncalves et al., 2008). A productive infection requires more, however, than a successful invasion of hepatocytes. It was recently shown that parasites lacking the sporozoite low complexity asparagine-rich protein (SAP1) were able to migrate and invade but failed to develop of a productive infection due to the repression of several genes required for efficient development in the liver (Aly et al., 2008).

6. Invasion: how to go in?

6.1. Moving junction formation

Invasion is a unique process tightly coupled to the sequential secretion of two types of apical organelles named micronemes and rhoptries. The micronemes are first discharging proteins thought to participate in gliding motility and host cell recognition (reviewed in Carruthers and Tomley, 2008) followed by the release from the rhoptries, club-shaped organelles with an anterior part called rhoptry 'neck' extending in the apical end.

In *T. gondii*, successful subcellular fractionation resulted in an enrichment in rhoptries, allowing the identification of more than 30 rhoptry proteins, some sequestered in the bulb (ROPs) and others located in the duct part of the rhoptry (RONs) (Bradley et al., 2005). Lipids are also known to be contents of these organelles (Foussard et al., 1991; Besteiro et al., 2008). Since many of the identified proteins are conserved across much of the phylum and are secreted during invasion, the rhoptries have long been suspected as playing a key role in the intracellular lifestyle of the Apicomplexa. However, it was only recently shown that their contribution to invasion is not restricted to providing building material for the developing PV; they are also involved in modifying of the host cell following invasion (Bradley et al., 2005; Bradley and Sibley, 2007; Boothroyd and Dubremetz, 2008).

Host cell invasion is exceptionally fast, taking about 10s, and it is intimately linked with gliding motility (see above). During this process host cell plasma membrane transmembrane (TM) proteins but not glycosylphosphatidylinositol (GPI)–anchored proteins are largely excluded from the newly formed PV, as shown for invasion of cells by *T. gondii* (Mordue et al., 1999) and of RBCs by *Plasmodium* merozoites (Aikawa et al., 1978). This remarkable vacuole remodelling remains a conundrum, but it is known to take place at the site of close attachment between the parasite and the host cell membrane, named the moving junction (MJ), suggesting that

molecules that build the MJ are involved in this molecular sieving. The term Moving Junction, coined by Aikawa et al. (1978) to describe "a zone of attachment between the erythrocyte and merozoite that moves along the confronted membranes to maintain its position at the orifice of the invagination" is a region of tight membrane contact (less than 6 nm) between the parasite and the host cell membranes, with the latter being markedly thickened. It begins as a cup covering the parasite apex and rapidly turns into a ring encircling the parasite, moving backwards relative to the parasite, and when entry is completed, fusion occurs at the posterior end of the parasite. The movement that propels the parasite into the nascent PV is possible because the proteins forming the MJ are probably connected to yet unidentified components of the actomyosin motor.

The molecular components of the MJ remained a mystery for a long time but recent reports highlighted an association between microneme and rhoptry proteins (Alexander et al., 2005). Some of these are hypothetical proteins restricted to Apicomplexa, suggesting that these parasites have developed a specific machinery for host cell invasion that has no counterpart in other cells. The cooperation between proteins of the micronemes and the rhoptries at the MJ is supported by several pieces of data published throughout the years. It was first shown that antibodies against the microneme protein PfAMA1 inhibit the committed attachment between *Plasmodium* merozoites and RBCs – the initial random surface attachment of merozoites to RBCs was not affected but the close junctional contact was absent (Mitchell et al., 2004). Then Mital et al. (2005), using an engineered *T. gondii* strain expressing less than 0.5% of TgAMA1, showed that this protein is not involved in gliding motility, or in the initial step of attachment, or in microneme release, but it is needed for an intimate attachment to the host cells. Finally, it was demonstrated that the rhoptry neck protein TgRON4 co-localizes with the MJ (Lebrun et al., 2005) where it associates with TgAMA1 (Alexander et al., 2005), and that TgAMA1 deletion has no effect on RON4 secretion but abolishes its recruitment at the MJ and blocks invasion. Moreover the presence of PfRON4 at the MJ of merozoites invading RBCs (Baum et al., 2008) and the association of PfAMA1 with PfRON4 in *P. falciparum*, suggest that the collaboration between micronemal AMA1 and rhoptry RON proteins is a conserved feature (Alexander et al., 2006).

The MJ complex is now known to contain other rhoptry proteins (RON2, RON4, RON5). They were identified in *T gondii* by pull-down experiments with anti-RON4 or anti-AMA1 antibodies, and cross-linking experiments during host cell invasion by this same parasite have demonstrated that this RON complex is probably pre-formed inside the parasite but only associates with AMA1, which is secreted first, upon discharge of the rhoptries onto the parasite's surface (Alexander et al., 2005). It is still unclear, however, how the complex is organized at the MJ.

As mentioned above, the MJ complex represents a stable frame at the cell surface onto which the parasite grabs to propel itself inside the cell using its gliding motion, implying that it is linked to the subpellicular motor of the parasite. In such a model AMA1, which shares homologies with TgMIC2 in its C-terminal domain, should interact with the glideosome to ensure translocation of the MJ during invasion. However, this protein does not possess the critical tryptophan in its C-terminus that appears necessary for its connection to the sub-membranous motor (Jewett and Sibley, 2003) and therefore an indirect interaction of TgAMA1 with other transmembrane MIC proteins such as TgMIC2 at the MJ seems more likely. This model also implies that the MJ is held at the host cell surface by interacting with a stable cytoskeletal structure, indirectly via association with integral host proteins linked to the cytoskeleton, or directly by interaction of the host cell subplasmalemmal cytoskeleton with the RONs complex inserted into the host plasma membrane. Since RON4 and RON5 contain no predicted TM domains, RON2, which is predicted to have two or three TM domains, may be responsible for such a function. This model is supported by electron microscopy of the MJ showing a thickening of the host cell membrane and a specific substructure (Aikawa et al., 1978), which suggests recruitment of proteins at this level. In this exciting scenario, the insertion of the parasite's own invasion apparatus into the host cell membrane would not only act as a grip and contribute to exclusion of cytoskeletal and TM proteins from the vacuole, but would also explain the large diversity of cells invaded by most Apicomplexa.

Interestingly, AMA1 and RONs proteins are present in all the genomes of Apicomplexa sequenced to date, with the exception of the *Cryptosporidium* genus, which displays a markedly distinct mode of zoite-host cell interaction. In the case of *Theileria*, no MJ is visualized during leucocyte invasion by sporozoites or merozoites but this process is considered as a zippering interaction driven by the host cell (Fawcett et al., 1984), and these sporozoites and merozoites are known to differ from classical apicomplexan zoites in many significant aspects (they have no clear apical complex or IMC and are non-motile). Nevertheless, in the tick vector the *Theileria* kinete possesses an apical complex and is motile, suggesting formation of a MJ.

Further studies are obviously necessary to dissect the functional significance of RONs at the MJ, since almost all data reported to date are derived from studies on only one stage of one species, *T. gondii*.

6.2. Secretion and post-secretory fate of MICs and ROPs: signalling issues

One of the most sophisticated features of invasion by Apicomplexa is the coordinated secretion of micronemes and rhoptries. As discussed above, microneme secretion is regulated by an increase in the cytoplasmic calcium that is released from intracellular stores in the parasite. Host cell calcium is dispensable, as microneme discharge can be artificially triggered by ionophores (Carruthers and Sibley, 1999) and occurs before the interaction with a host cell since it is required for gliding motility (Lovett and Sibley, 2003). On the other hand, secretion of rhoptries cannot be mimicked in the absence of host cells, suggesting that it is dependent on intimate contact of the parasite with the host cell membrane.

As the parasite penetrates the host cell, most MIC proteins (except AMA1) are excluded from entering the vacuole and are progressively capped behind the MJ, remaining confined to the portion of the parasite that still protrudes from the host cell. In contrast, rhoptry proteins reach at least four destinations: (i) several RONs remain associated with the MJ; (ii) some ROPs end up in the PV, (iii) others associate with the PVM, and (iv) another group of ROPs are found beyond the PVM, in the host cell nucleus (Boothroyd and Dubremetz, 2008).

How the different proteins reach their final destination is unknown. It may be due to the association with lipids that are sometimes visualized using electron microscopy as membrane whorls inside the rhoptries (Nichols et al., 1983). These lipid vesicles (termed e-vacuoles) are secreted in the host cell cytoplasm and then fuse with the parasite-containing vacuole, in a mechanism blocked by cytochalasin D (Hakansson et al., 2001). While the signalling leading to microneme exocytosis and its direct consequence, i.e. gliding motility, is rather well known (discussed above), the trigger for rhoptry exocytosis is entirely distinct and has not been elucidated. It is therefore unlikely that microneme and rhoptry neck fuse before exocytosis, as is sometimes suggested in the literature.

7. Conclusion

This short review aims to highlight the questions that still remain concerning every step of the apicomplexan lytic cycle and the most recent findings regarding the proteins and mechanisms involved (check Supplementary Table S1 for a summary). It is still not possible to fully understand how these parasites exploit novel processes and proteins in order to be efficient pathogens, and therefore more research is needed. Only in this way will the parasites' "Achilles' heels" be exposed and the fight against these pathogens become more efficient.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.10.007.

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