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Microreview

The moving junction of apicomplexan parasites: a key structure for invasion

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Summary

Most Apicomplexa are obligate intracellular parasites and many are important pathogens of human and domestic animals. For a successful cell invasion, they rely on their own motility and on a firm anchorage to their host cell, depending on the secretion of proteins and the establishment of a structure called the moving junction (MJ). The MJ moves from the apical to the posterior end of the parasite, leading to the internalization of the parasite into a parasitophorous vacuole. Based on recent data obtained in *Plasmodium* and *Toxoplasma*, an emerging model emphasizes a cooperative role of secreted parasitic proteins in building the MJ and driving this crucial invasive process. More precisely, the parasite exports the microneme protein AMA1 to its own surface and the rhoptry neck RON2 protein as a receptor inserted into the host cell together with other RON partners. Ongoing and future research will certainly help refining the model by characterizing the molecular organization within the MJ and its interactions with both host and parasite cytoskeleton for anchoring of the complex.

Host invasion by Apicomplexa

The phylum Apicomplexa comprises parasitic pathogens responsible for many veterinary or human diseases; these include the malaria-causing parasite (*Plasmodium* spp.), several major animal pathogens (e.g. *Eimeria* spp., *Theileria* spp., *Babesia* spp., *Neospora caninum*) and the causative agents of toxoplasmosis (*Toxoplasma gondii*) and cryptosporidiosis (*Cryptosporidium* spp.). Its members have complex life cycles, with several developmental stages, but most of them are, at one point or another, obligate intracellular parasites. Quite obviously, the intracellular habitat offers some major advantages to the parasite in terms of evading detection and destruction by the host's immune system. Different apicomplexan parasites specifically invade erythrocytes, lymphocytes, macrophages or cells of the digestive tract in various animal species, yet they share a conserved mode of invasion. Successful invasion by Apicomplexa involves a specific stage of the parasite (also called zoite), which bears a specialized complex of secretory organelles. Sequential secretion of these apical organelles, termed micronemes and rhoptries (Carruthers and Sibley, 1997), allows the motility of the parasite and the attachment to their host cell (by the use of micronemal proteins) and the subsequent penetration and establishment of the parasite in a membrane-bound structure called the parasitophorous vacuole (or PV, by the use of rhoptry proteins) (Fig. 1A). Rhoptries are club-shaped organelles containing two sets of proteins segregated either in the neck (rhoptry neck proteins or RONs that are often conserved among Apicomplexa and of which several are involved in the initial steps of invasion) or in the posterior bulb (rhoptry bulb proteins or ROPs, which tend to possess no orthologues across each of the genera, and are involved in later stages of invasion and parasite establishment) (Bradley *et al.*, 2005).

Apicomplexan zoites are polarized cells that start to penetrate their host cells with the apical end of the parasite leading. One central structure formed during invasion is the moving junction (MJ), a tight connection between zoite and host cell's plasma membranes that starts at the apical pole and moves progressively to the posterior end of the parasite as it enters the cell (hence the name 'moving junction'). The MJ is important for a successful invasion as it serves as a support to propel the parasite into the PV, but is also thought to be involved in the formation and in defining the biochemical composition of the PV membrane. For instance, the molecular complex formed by MJ proteins could participate in the selective sieving of host plasma membrane proteins that will be incorporated in the PV membrane, from which type I

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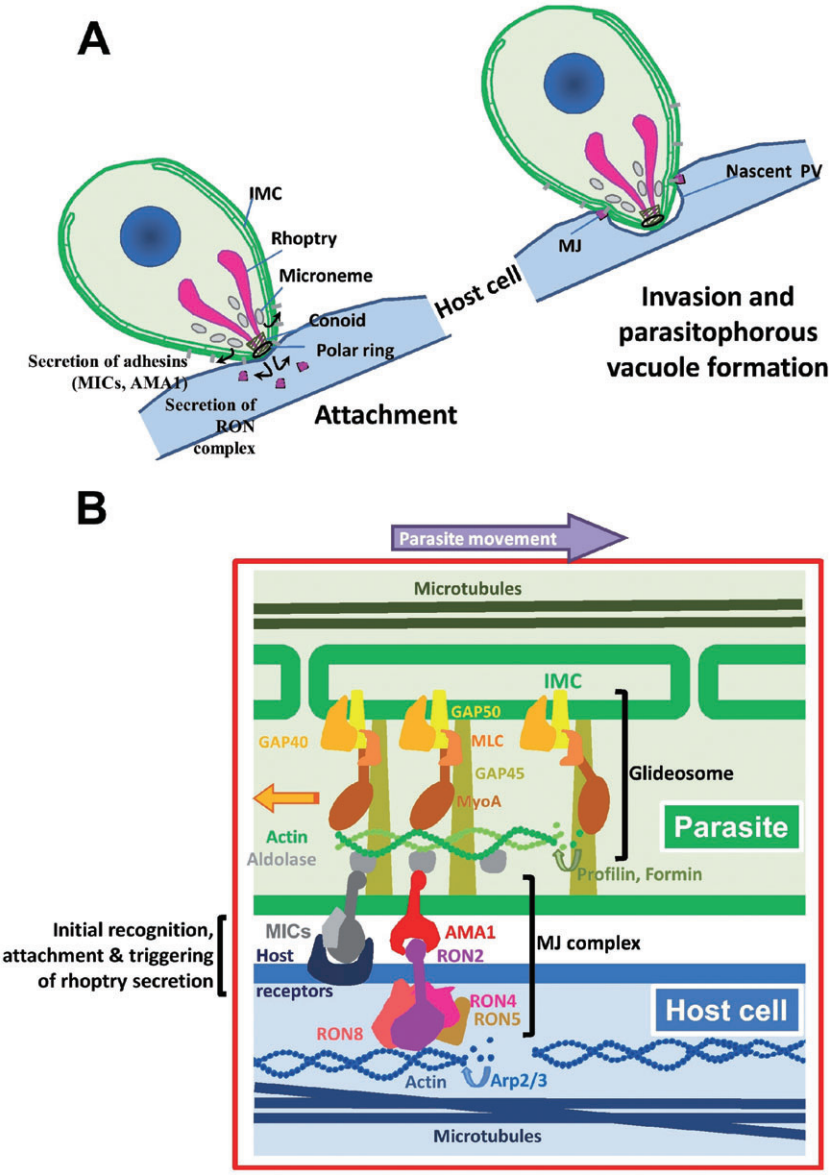

Fig. 1. The apicomplexan invasion machinery.
A. Initial attachment and invasion steps of a typical apicomplexan zoite stage. The zoite first binds to its host cell using secreted microneme proteins and secretes rhoptry material, including components of the MJ (left). Active penetration of the parasite inside its host cell after MJ formation as the PV forms (right).
B. Detail of the MJ annotated in A. Current model of apicomplexan motility machinery, the MJ complex and their respective links with host and parasite cytoskeletons.

transmembrane proteins are excluded, while GPI-anchored or raft-associated multipass transmembrane proteins enter the vacuole (Mordue *et al*., 1999; Charron and Sibley, 2004). This will prove crucial for later parasite development as it contributes to the non-fusogenic nature of the PV and would prevent its fusion with host lysosomes (Morisaki *et al*., 1995).

The morphological features of the MJ have been described more than 30 years ago in *Plasmodium* (Aikawa *et al*., 1978), yet only very recently tools have become available to decipher the MJ structure at the molecular level.

The molecular composition of the MJ is globally conserved among Apicomplexa

Molecular characterization of the junction has started quite recently. Immunoprecipitation experiments in *Toxoplasma* with specific antibodies have identified a macromolecular complex comprising rhoptry neck proteins RON2, RON4, RON5 and RON8 (Alexander *et al*., 2005; Lebrun *et al*., 2005; Besteiro *et al*., 2009; Straub *et al*., 2009). These RON proteins are secreted at the apical tip of the parasite and colocalize with the MJ during invasion, forming a characteristic ring-shaped

structure. Alexander *et al*. demonstrated that the micronemal Apical membrane antigen 1 (AMA1) is also part of the MJ macromolecular complex that can be isolated *in vitro* and, while the protein is secreted at the apical tip and localizes to the entire surface of *T. gondii* tachyzoites on extracellular parasites, it can also be found at the MJ during invasion (Alexander *et al*., 2005). Overall, this suggested that RONs and MICs, although secreted from different organelles, collaborate to establish the MJ; yet the asynchronous biosynthesis of RONs and MICs would preclude the RONs/AMA1 interaction early in the biosynthetic pathway (Besteiro *et al*., 2009).

The RONs/AMA1 *in vitro* interaction has been confirmed for *Plasmodium* (Alexander *et al*., 2006; Narum *et al*., 2008; Cao *et al*., 2009; Collins *et al*., 2009) and the presence of both *Pf*RON4 and *Pf*AMA1 at the MJ has been recently demonstrated (Riglar *et al*., 2011). Most of the MJ complex proteins are conserved among Apicomplexa (Table 1). Indeed, although the apicomplexan genome sequencing projects differ considerably in terms of completion or sequence coverage, RON2, RON4, RON5 and AMA1 orthologues can be found in the genomes of *Plasmodium*, *Toxoplasma*, *Neospora*, *Eimeria*, *Theileria* and *Babesia* species. This suggests a ubiquitous and conserved function of the MJ within these parasites. One notable exception is RON8 that seems to be restricted to *Toxoplasma*, *Neospora* and *Eimeria* (Table 1), which could highlight a more specialized function for this protein in the Coccidia. Intracellular developmental stages of Apicomplexa usually occupy a vacuolar space lined by a membrane (the PV), primarily derived from the plasmalemma of the host cell (Suss-Toby *et al*., 1996), but significantly modified upon passing through the MJ. It is important to note that the conservation of MJ components among Apicomplexa seems related to their mode of interaction with the host cell: all intracytoplasmic PV-forming parasites, whether they develop within the PV (*Toxoplasma*, *Neospora*, *Plasmodium* and *Eimeria*) or eventually escape to the cytosol shortly after invasion (such as the Piroplasmidae *Theileria* and *Babesia*) possess the MJ components. On the contrary, *Cryptosporidium* that remains extracytoplasmic, as it is wrapped into a PV resting on a pedestal of actin filaments at the apical surface of epithelial cells of the gut (Clark and Sears, 1996), does not possess any orthologue of the MJ partners in its genome.

Formation and organization of the MJ, its components and their interactions

Of all MJ components, AMA1 is clearly the best characterized so far. AMA1 was first identified in *Plasmodium knowlesi* (Deans *et al*., 1982) and was shown to be relocalizing from micronemes to the parasite surface. AMA1 is

Table 1. Members of the MJ complex and their isoforms that can be found in the genomic databases of apicomplexan species.

	Plasmodium falciparum	*Toxoplasma gondii*	MS	EST	*Neospora caninum*	EST	*Eimeria tenella*	*Cryptosporidium parvum*	*Theileria annulata*	*Babesia bovis*
RON2	PF14_0495	TGME49_100100	✓	✓	NCLIV_064620	✓	ETH_00012760	–	TA19390	XP_001608815
RON2 iso1	–	TGME49_094400	–	✓	NCLIV_001400	–	ETH_00013625	–	–	–
RON2 iso2	–	TGME49_065120	–	✓	NCLIV_040110	✓	ETH_00028240	–	–	–
RON4	PF11_0168	TGME49_029010	✓	✓	NCLIV_030050	–	ETH_00022715	–	TA13245	XP_001612212
RON4 iso1	–	TGME49_053370	✓	✓	NCLIV_007800	✓	–	–	–	–
RON5	MAL8P1.73	TGME49_111470	✓	✓	NCLIV_055360	✓	ETH_00015305	–	TA16660	XP_001611063
RON8	–	TGME49_106060	✓	✓	NCLIV_070010	✓	ETH_00031645/ETH_00031650	–	–	–
AMA1	PF11_0344	TGME49_055260	✓	✓	NCLIV_028680	✓	ETH_00007745	–	TA02980	XP_001611043
AMA1 iso 1	–	TGME49_115730	–	–	NCLIV_058410	–	ETH_00004860	–	–	–
AMA1 iso 2	–	TGME49_100130	–	✓	NCLIV_064590	–	ETH_00017730	–	–	–
Source database:	Plasmodb.org	Toxodb.org			Toxodb.org		Genedb.org		Genedb.org	GenBank

The MS and EST columns reflect the existence of proteomic mass spectrometry data and expressed sequence tags (cDNA sequences), respectively, suggestive of an expression of these genes.

known to play a central role in invasion by apicomplexan parasites. For instance, it appears to be essential for invasion of *Plasmodium* blood stages, as no knock out mutants can be obtained (Triglia *et al*., 2000), and conditional *T. gondii* AMA1 null mutant tachyzoites are unable to invade host cells although they correctly attach to the host cell (Mital *et al*., 2005). Moreover, in *Plasmodium* (merozoite or sporozoite), *Toxoplasma*, *Babesia* and *Neospora*, antibodies against AMA1 block parasite invasion (Hehl *et al*., 2000; Hodder *et al*., 2001; Silvie *et al*., 2004; Zhang *et al*., 2007; Montero *et al*., 2009) and so does the specific AMA1-binding R1 peptide, a peptide obtained by screening a phage-display library (Harris *et al*., 2005). Once secreted at the surface of the parasite, AMA1 is a type I integral membrane protein, with a short cytosolic region and the bulk of the protein forming the ectodomain. Recent structural studies on *Plasmodium* and *Toxoplasma* AMA1 (Pizarro *et al*., 2005; Crawford *et al*., 2010) have confirmed that they are structurally conserved: the ectodomain is composed of three architectural domains and contains a conserved hydrophobic trough surrounded by polymorphic loops. However, the understanding of the precise role of AMA1 in invasion and of the mode of action of anti-AMA1 invasion-inhibitory antibodies has remained incomplete. In *Plasmodium falciparum*, two recent works (Collins *et al*., 2009; Richard *et al*., 2010) have shed new light on the mechanisms of AMA1 function and antibody-mediated inhibition of invasion. They showed that an antibody directed against AMA1, or the AMA1-binding R1 peptide, inhibited invasion by preventing *Pf*AMA1 from interacting with the *Pf*RON complex, without defining which RON is involved in this interaction. Subsequent work demonstrated that the R1 peptide inhibited directly an interaction between *Pf*AMA1 and *Pf*RON2 (Lamarque *et al*., 2011). Furthermore, live video microscopy of invasion of *P. falciparum* merozoites in the presence of R1 peptide revealed that the primary reorientation step (allowing contact of the apical pole with the erythrocyte surface), the attachment and oscillatory deformation of the red blood cell membranes are not affected, but that parasites fail to progress further (Treeck *et al*., 2009), an observation that could be explained by the lack of MJ formation.

RON proteins do not bear recognizable domains or motifs that could suggest a particular function or molecular interaction. It has been shown in *Toxoplasma* that RONs are subjected to proteolytic maturation (Besteiro *et al*., 2009; Straub *et al*., 2009) and processed in the pre-rhoptries, which is likely important for their function, but is not a prerequisite to their interaction, as non-matured proteins were found to be interacting *in vitro* (Besteiro *et al*., 2009). RON4, RON5 and RON8 are putative soluble proteins, while RON2 is predicted to have between two and four transmembrane domains depending on the software used. Using antibody-loaded host cells, we have shown that the RONs pertaining to the MJ are exposed on the cytosolic side of the host cell during invasion, which implies that they are secreted inside the host cell by the parasite (Besteiro *et al*., 2009). Straub *et al*. also obtained data for *Tg*RON8 that are in accordance with this model (Straub *et al*., 2009).

Among the MJ proteins, only RON2 and AMA1 have been shown to interact directly so far (Besteiro *et al*., 2009). Two recent studies defined the C-terminal part of *Tg*RON2 as the domain of interaction with *Tg*AMA1 (Lamarque *et al*., 2011; Tyler and Boothroyd, 2011). These findings, together with the results showing that the N-terminal part of RON2 is exposed on the cytosolic side of the host membrane (Lamarque *et al*., 2011), confirm that *Tg*RON2 is a *bona fide* transmembrane protein. More importantly, a short *Tg*RON2 sub-region of only 54 amino acids was found to interact with *Tg*AMA1 and block invasion, but not attachment, in a *Tg*AMA1-dependent manner, demonstrating that the AMA1/RON2 interaction occurs during the invasion of host cell by *T. gondii* tachyzoites. Interestingly, the interaction and its function are also conserved in *P. falciparum*, although both proteins display a significant degree of sequence variation between these two apicomplexan genera (Lamarque *et al*., 2011). It thus reflects a co-evolution of the AMA1 protein and of its RON2 partner, and points towards the important functional role of this interaction in Apicomplexa.

Overall, AMA1 being a transmembrane protein on the parasite side and RON2 a transmembrane protein on the host cell side, we have proposed a model where the parasite would be inserting its own receptor (RON2 and associated RON proteins) for AMA1 to create the MJ [(Besteiro *et al*., 2009; Lamarque *et al*., 2011), Fig. 1B]. The characterization of the RON2/AMA1 interaction is thus a first step towards dissecting the specific interaction between members of the MJ protein complex, but their interplay with the other RONs located within the host cell's cytoplasm is still largely unknown.

Anchoring of the MJ on the host cell's side

The MJ, although moving (hence its name) along the parasite cell body, remains static on the host cell side and supposedly requires some kind of anchoring to the host cell membrane or the underlying cytoskeleton. Apicomplexan parasite entry is a rapid process (less than a minute) and, unlike cell invasion by pathogenic bacteria or viruses, it is mainly driven by the pathogen and requires a limited contribution of the host cell. For example, it was believed that host cell actin cytoskeleton had no contribution to the invasion process (Morisaki *et al*., 1995). While it is still undisputed that parasite motility is the main driving

force behind the invasion event, recent studies however, are pointing towards a contribution of host cell components in this process. Using *T. gondii* tachyzoites and *Plasmodium berghei* sporozoites as models of their study, Gonzalez *et al*. (Gonzalez *et al*., 2009) have localized actin and actin nucleators such as Arp2/3 at the MJ and suggested that *de novo* polymerization of host actin at the entry site was essential to invasion. In red blood cells (the hosts of *Plasmodium* and *Babesia* merozoites), the stability of the actin cytoskeleton is controlled by tropomodulin, tropomyosin, adducin and dematin, but no actin nucleation factors have been described; interactions between these proteins and MJ proteins remain to be assessed. Parasite-derived actin nucleators were initially thought to be involved in direct host actin regulation, for instance *Toxoplasma* toxofilin can stabilize F-actin through a filament end-capping activity *in vitro* (Poupel *et al*., 2000) and appears to be secreted into host cells during invasion; however, it seems to play little role on host actin as *toxofilin* knock out tachyzoites can still invade their host efficiently (Lodoen *et al*., 2010).

One can imagine that manipulation of host actin nucleators could be exerted by upstream parasitic regulators and MJ proteins would be potential candidates for this, although such an interaction has yet to be demonstrated. *Tg*RON8, of which the C-terminus was found to be associated with the membrane/cytoskeleton in a mammalian heterologous expression system (Straub *et al*., 2009) could be such a candidate, but it is not conserved in all Apicomplexa. Besides actin microfilaments, which are most concentrated just beneath the cell membrane, another constituent of the host cell cytoskeleton has been suggested to be involved in an interaction with invading apicomplexan parasites: the microtubule network. Indeed, occasional association of host microtubules with *Toxoplasma* MJ has been described and an early stabilizing role for parasite entry has been proposed: integrity of the host microtubule cytoskeleton appears to render invasion by *Toxoplasma* tachyzoites more efficient (but is not essential for it) (Sweeney *et al*., 2010).

Of course, the cortical host cell cytoskeleton also represents a physical barrier for parasite entry and before the MJ is established and anchoring for the entering parasite is triggered, it is likely that the host cytoskeleton must be destabilized locally to allow the initiation of entry. How these antagonistic destabilizing/stabilizing functions are achieved in such a short period of time (seconds) and what are the parasitic factors involved are still a mystery.

A link with the motor complex on the parasite side through AMA1?

Apicomplexan parasites share a substrate-based motility mechanism termed gliding. This locomotion requires binding to a substrate by the use of adhesins expressed by the parasite on its surface, while the propulsive force is provided by an acto-myosin motor located between the parasite plasma membrane and a network of membrane sacs known as the inner membrane complex (IMC, Fig. 1). This motor complex has been termed the glideosome and particularly well characterized in *Toxoplasma* (see Daher and Soldati-Favre (2009) for a review). It consists of Myosin A, a class XIV myosin motor, associated with myosin light chain, itself linked to the gliding-associated proteins 45 [GAP45 (Gaskins *et al*., 2004)], a protein anchored to the plasma membrane and IMC via its N- and C-terminal ends respectively (Frénal *et al*., 2010). Myosin is a stationary motor acting as a swinging lever arm on F-actin filaments, the polymerization of which is crucial for the motility of the parasite. Apicomplexa lack the actin nucleator protein complex Arp2/3, but important contributors to parasitic actin polymerization include profilin, which is essential for motility and host cell invasion by *T. gondii* tachyzoites (Plattner *et al*., 2008) and formin, which has been claimed to be specifically recruited near the MJ during invasion by *P. falciparum* merozoites (Baum *et al*., 2008), while it was found to be evenly distributed under the plasma membrane in *T. gondii* (Daher *et al*., 2010). The glycolytic enzyme aldolase provides a link between F-actin and the surface-exposed transmembrane protein MIC2 (Jewett and Sibley, 2003; Starnes *et al*., 2009) (Fig. 1B). This way, MIC2 [or the thrombospondin-related anonymous protein (TRAP) in *Plasmodium*] allows the coupling of host cell recognition with the parasitic acto-myosin motor system. Motility and invasion of host cells depend on the treadmilling of the adhesins fixed on host cell receptors (or substrate in the case of motility), towards the posterior end of the parasite as it penetrates. Binding to aldolase is mediated by the C-terminal part of the TRAP/MIC2 proteins: series of acidic amino acids (aspartic and glutamic acids) and a tryptophane residue are of particular importance for binding (Buscaglia *et al*., 2003; Starnes *et al*., 2006).

Until recently, the link between the acto-myosin motor of the parasite and the MJ needed to mediate the posterior translocation of the MJ complex during invasion was not known, although recent imaging of invading *Plasmodium* merozoites showed a ring of parasite actin following the MJ (Riglar *et al*., 2011). The C-terminal part AMA1 has some similarities with the cytoplasmic tail of TRAP family proteins, both in *Toxoplasma* or *Plasmodium*: it is rich in acidic residues and bears a tryptophan (although less terminal than for the TRAP/MIC2 counterpart, Fig. 2), yet immunoprecipitation from parasite extracts never recovered aldolase as a binding partner for AMA1. An elegant *in vivo* complementation of function approach with mutant versions of AMA1 has nonetheless shown that a FW motif and several acid residues from the C-terminal region of

Fig. 2. Alignment of the acidic amino acids-rich C-terminal end of TRAP/MIC2 family members and AMA1. GenBank accession numbers: TgAMA1 (ACM44988), TgMIC6 (AAD28185), TgMIC2 (AAB63303), PfTRAP (XP_001350088), PfCTRP (XP_001351221), PfmTRAP (XP_001347565), PfTLP (CAG25403), PfAMA1 (XP_001348015). The arrow points the conserved tryptophan that is involved in the binding of aldolase.

*Pf*AMA1 are essential for invasion by *Plasmodium* (Treeck *et al*., 2009). Moreover, it has now been demonstrated that recombinant *Tg*AMA1 can bind to aldolase in an *in vitro* pull-down assay and that mutation of C-terminal FW residues or acidic amino acids of *Tg*AMA1 prevents the binding and inhibits invasion of the host cell by *Toxoplasma* (Sheiner *et al*., 2010), reinforcing the idea that AMA1 could be the link between the MJ and the glideosome (Fig. 1B). Biochemical and mutational analyses have delineated specific residues in aldolase critical for linking the cytoplasmic tail of MIC2 to the cytoskeleton and separated this function from residues participating in glycolysis (Starnes *et al*., 2009). Unexpectedly, it was found that the aldolase–MIC2 link is dispensable for motility and the attachment step, while it is required for the subsequent step of invasion. It would be interesting to assess if the step that is affected is the MJ formation and if these mutants are also impaired in binding to AMA1.

A puzzling feature of AMA1 is its distribution all over the parasite surface during invasion. It is not restricted to the MJ (Howell *et al*., 2005), which renders its labelling and precise localization at the MJ difficult (Alexander *et al*., 2005). A minimal amount of AMA1 is apparently needed to mediate invasion: the non-induced conditional *AMA1* knock out mutant, albeit expressing only ~ 10% of native AMA1 levels, is still invading with comparable efficiency as the wild-type strain (Mital *et al*., 2005). Only a small part of AMA1 might be associated with the MJ and the glideosome (which is potentially why the aldolase–AMA1 interaction has never been detected from cellular extracts). However, the role of the AMA1 pool located at the surface of the parasite plasma membrane is, at the moment, unknown. The cytoplasmic tail of AMA1 can be phosphorylated (Treeck *et al*., 2009) and thus, as suggested by others (Sheiner *et al*., 2010), differential phosphorylation within the AMA1 pool might induce a conformational change in the extracellular domain resulting in different functional activations. In addition, *Tg*AMA1 cleavage by the intramembrane rhomboid 4 protease has been shown to be essential for further intracellular parasite development (Santos *et al*., 2011), highlighting a possible role of AMA1 in co-ordinating the start of intracellular replication with invasion.

One MJ complex or several?

The fact that the MJ complex is at the same time providing the key and the lock for parasite entry, could explain in a way the ability of *Toxoplasma* to invade so many different cell types. However, other apicomplexan species or life stages are not so ubiquitous in their invasion potential, hence there must be some kind of specificity driving the establishment of the junction. It has been recently shown that the secretion of rhoptries, a key determinant for MJ formation, is dependent on the recognition of host cell receptor(s) by microneme proteins (Singh *et al*., 2010). However, using a cell reporter system for secretion, Tyler and Boothroyd recently confirmed that the formation of the MJ is not a prerequisite for injection of rhoptry bulb proteins inside the host cell (Tyler and Boothroyd, 2011).

Database searches have revealed that MJ proteins orthologues were presents in PV-forming Apicomplexa (Table 1), but it seems that not all MJ complexes might be identical between species, as illustrated by the presence of RON8 exclusively in Coccidia. Variation can also be expected between the life stages within the same species. To this regard, the case of *Theileria* is particularly interesting, as the merozoite and sporozoite stages are non-motile (with no micronemes), and require neither apical attachment to the host cell nor apical organelle secretion for invasion. Instead, they enter their host cell by a zippering process, which also leads to the formation of a PV (Shaw, 2003). Nevertheless, the genes encoding proteins of the gliding machinery are present in the *Theileria* genome and so are the components of the MJ (Table 1). Quite simply, it might be that this machinery is needed by another life stage, such as the kinetes, which egress the gut epithelial cells and invade the salivary glands in the tick using gliding motion.

Database searches also reveal a fact that has not been explored, which is the presence of paralogues of MJ complex members AMA1, RON2 and RON4 in Coccidia such as *Toxoplasma*, *Neospora* and *Eimeria*. *Tg*RON4 has one additional copy, and strikingly the two interactors *Tg*AMA1 and *Tg*RON2 have two extra copies each. It is not known whether these isoforms are expressed (but several seem to be, according to the mass spectrometry

or EST data, Table 1) and at which stage, and also if they localize to the MJ during invasion, but they certainly deserve to be studied. Indeed, the presence of these isoforms could reflect the formation of an alternative MJ complex with a different composition, which could be stage-specific, or the possibility to build a heterologous MJ complex. Regarding this, mass spectrometry data from the initial isolations of the MJ protein complex in *Toxoplasma* tachyzoites systematically and consistently recovered peptide sequences from AMA1, RON2 and RON4, but not their isoforms, suggesting a rather homogenous composition of the MJ complex in this life stage. Differential proteomic analysis of *Eimeria* life stages detected two different AMA1 isoforms expressed with RON4 only in merozoites and with RON5 only in sporozoites, respectively (Lal *et al*., 2009) that could reflect a stage-specific MJ composition.

Finally, it has been suggested that the MJ machinery can be used for the process of egress (Alexander *et al*., 2005). This process, by which intracellular parasites leave the PV after replication to invade new cells or a new host, is only partially understood. However, it is unlikely that a MJ similar to the one required for entry is formed, as rhoptry secretion has never been reported at this stage and AMA1 is not detected on the parasite surface.

Remaining questions

Although significant progress has been made over the last few years to elucidate the molecular composition and the organization of the MJ, many questions remain to be addressed.

The link between the RONs and the host cell cytoskeleton has not been established and the identification of host cell partners has to be pursued. On the other side, the link between AMA1 and the parasite motor complex, although demonstrated *in vitro*, still has to be investigated *in vivo*. In relation to this, understanding when the MJ components assemble and how the rhoptry-derived material forming the MJ is secreted and inserted into the host cell remains a major black box, which is still difficult to tackle experimentally but deserves a particular attention.

The potential existence of host cell- or stage-specific MJ complexes remains to be shown, but it could give insights into the specificity of the AMA1/RON2 interaction, especially if non-interchangeable AMA1/RON2 couples are identified. Given that the MJ is likely essential for the invasion and survival of the Apicomplexa intracellular stages, and given the key interaction of RON2/AMA1 within the MJ, identifying precisely the residues involved in this interaction would clearly open interesting perspectives for a new way of interfering with parasite invasion, with an obvious therapeutic potential against pathogenic Apicomplexa.

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