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Host cell invasion by apicomplexans: what do we know?

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Apicomplexan zoites enter host cells by forming and actively moving through a tight junction (TJ) formed between the parasite and host cell surfaces. Although the TJ was first described decades ago, its molecular characterization has proved difficult mainly because of its transient existence during an internalization process that lasts only seconds. In the past 7 years, work has led to a model of the TJ in which the association between AMA1 and RON proteins structures the TJ and bridges the cytoskeletons of the two cells. However, more recent work questions this view. Here, we critically discuss the current model and speculate on alternative models of the AMA1–RON association and of the apicomplexan TJ.

The apicomplexan tight junction

Apicomplexans constitute a large phylum of protists, most of which are parasites. The best known apicomplexan parasites of humans are *Plasmodium* and *Toxoplasma*, the agents of malaria and toxoplasmosis, respectively. They multiply inside host cells and their extracellular forms are polarized and motile cells, termed zoites, which possess a submembrane actomyosin motor. Zoites invade host cells by a rapid process powered exclusively by their motor [1]. After binding to the host cell, in a way that depends on the particular zoite–cell combination, the zoite forms a tight junction (TJ) between its apical tip and the host cell [2]. The TJ is thought to connect the cortical actins in the two cells and thus to serve as a stable anchor for myosin-dependent zoite traction into a parasitophorous vacuole (PV) [3]. Another function of the TJ is to selectively exclude the transmembrane proteins in the host cell membrane that invaginates during zoite internalization and becomes the vacuole membrane, a process that might play a role in preventing the fusion of the vacuole to host cell endosomal compartments [4]. Once inside the safe PV niche, the zoite can differentiate into multiple new zoites that eventually egress out of the host cell to infect new cells.

The AMA1–RON model of the apicomplexan TJ

Identifying the components of the TJ has been a major goal ever since the TJ was observed for the first time more than 30 years ago when examining *Plasmodium* merozoite invasion of erythrocytes [2]. Evidence has progressively mounted in recent years indicating that the TJ essentially consisted of interactions between two parasite proteins that are conserved in apicomplexans: AMA1 (apical membrane antigen 1) [5], a transmembrane protein at the parasite surface, and RON2 (rhoptry neck protein 2) [6], a protein inserted in the host cell membrane. It was initially found that a set of RON proteins specifically marked the TJ during cell invasion by the *Toxoplasma* tachyzoite, and that the TJ-associated RON proteins formed a complex with AMA1 in tachyzoite extracts [7,8]. The AMA1–RON complex, which contains at least RON2, RON4 and RON5 and in which AMA1 directly binds RON2 [9–11], was also pulled down from extracts of *Plasmodium* merozoites [12–16] and *Neospora* tachyzoites [17]. Studies then showed that peptides or antibodies that inhibited the AMA1–RON2 association also prevented host cell invasion by the *Plasmodium* merozoite and the *Toxoplasma* tachyzoite [9–11,15,16,18,19]. Furthermore, the AMA1-binding region of RON2 in *Toxoplasma* was mapped to a loop constrained by a disulfide bridge and the crystal structure of the interaction was solved [20]. It revealed that the RON2 loop penetrates deep within a hydrophobic groove in AMA1, suggesting that the association might withstand mechanical forces. Additionally, an extension in the loop of *Plasmodium* RON2 mirrors an extension in the *Plasmodium* AMA1 groove, providing evolutionary evidence for the importance of the interaction. Finally, an association was reported between the cytoplasmic tail of AMA1 and aldolase [19,21], currently described as a bridge between the cytoplasmic tails of parasite proteins and F-actin [22]. Therefore, the view that the AMA1–RON2 complex with a 1:1 stoichiometry is the zoite–cell link that ensures apicomplexan traction into the host cell has been widely accepted [23–32]. This model of a TJ composed of only parasite proteins was indeed attractive because it provided a rationale for the capacity of many apicomplexan zoites to enter virtually any nucleated cell.

Inconsistencies with the model

Recent data question the view that the AMA1–RON association mediates traction in the TJ. First, immunolocalizations in the *Toxoplasma* tachyzoite and the *Plasmodium* merozoite show that, whereas the secreted RONs constantly and precisely mark the TJ in a ring-like manner, the secreted AMA1 mostly covers the entire extracellular surface of the invading zoite. Selected immunofluorescence images have been reported [7,19,33] that suggest the presence of some AMA1 at the TJ, but they do not

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distinguish between AMA1 truly embedded in the TJ or merely present on the folded parasite membrane in the plane of the constricted area of the zoite. In addition, new immunolocalizations [34] indicate that although some invading *Toxoplasma* tachyzoites display a minor enrichment of AMA1 at the TJ, most do not. Clearly, the immunostainings of AMA1 and the RONs in invading zoites published so far, although suggestive of some occasional overlap, are not proof of a TJ structured by AMA1–RON complexes.

To date, the sole line of functional evidence favoring the accepted model is the inhibition of zoite invasion by antibodies or peptides that prevent the formation of the AMA1–RON complex *in vitro* [9–11,15,16,18,19]. This has been interpreted as a demonstration that the complex was crucial for invasion [32]. However, videomicroscopic examinations reported that *Plasmodium* merozoites that failed to invade erythrocytes in the presence of a complex-inhibitory AMA1-binding peptide [16,18] still vigorously pulled against the cell surface, which indicates that impeding AMA1–RON interactions still allows traction to be exerted by the parasite. Furthermore, an AMA1 antibody and a RON2 peptide that independently prevent complex formation were found to impair merozoite invasion at distinct steps of the process, as the AMA1 antibody, but not the RON peptide, also blocked secretion from the rhoptry organelles of the zoite [19]. Therefore, the complex-inhibitory antibodies or peptides might not impede zoite invasion only, or even primarily, by impairing complex formation.

Further doubts were cast on the role of the AMA1–RON 1:1 complex in TJ formation with the recently described phenotypes of various AMA1- and RON4-deficient zoites [34]. The same *AMA1* conditional knockdown mutation in *Plasmodium berghei*, which reduced AMA1 to undetectable levels, had no effect on *Plasmodium* sporozoite invasion of hepatocytes but prevented *Plasmodium* merozoite invasion of erythrocytes. In sporozoites, although the absence of AMA1 did not impair invasion, a partial decrease in RON4 was sufficient to reduce invasion. In *Toxoplasma* tachyzoites, decreasing AMA1 to undetectable levels diminished invasion frequency 10-fold [35] and yet still allowed the formation of a normal ring of RONs and a functional TJ by the invasive tachyzoites [34]. Therefore, genetic evidence so far favors the view of a zoite-dependent contribution of AMA1 during invasion and, at least in part, dissociated contributions of AMA1 and the RONs at the TJ.

In agreement with this, AMA1 appears to play a role upstream of TJ formation during zoite binding to the host cell. Earlier work has presented indirect evidence that *Plasmodium* AMA1 binds the erythrocyte surface [36–38]; that antibodies to *Plasmodium* AMA1 prevent the intimate attachment of the merozoite and erythrocyte membranes [39]; and that the lack of AMA1 in the *Toxoplasma* tachyzoite impairs the formation of intimate contacts with the host cell membrane following an initial, distant attachment step [35]. Recent work [34] has shown that AMA1-depleted *Toxoplasma* tachyzoites tended to adhere only via their anterior pole and to adopt an upright position, whereas the wild type adhere via their entire body in a flat position. These data point to a role of AMA1 in mediating intimate attachment between the zoite and host cell membranes, independently of the RONs. In *Plasmodium*, AMA1 might be important for stabilizing the pear-shaped *Plasmodium* merozoite in the reoriented position relative to the erythrocyte, whereas the elongated and highly flexible sporozoite might not need AMA1 to be correctly positioned for invasion. Importantly, AMA1, although mediating a basic function of intimate attachment of the zoite and host cell membranes, might ultimately confer different zoite orientations relative to the host cell depending on the zoite–cell combination and possibly on the protein surface expression pattern, for example throughout the zoite length or restricted to the anterior pole.

Valuable information on AMA1 function might also come from *Theileria*, an apicomplexan that cycles between a tick and mammalian host. The *Theileria* zoites that infect mammalian cells (the merozoites that invade erythrocytes and the sporozoites that invade leukocytes) are not motile and invade by a zipper mechanism, not by forming a TJ. The *Theileria* genome contains the *AMA1* and *RON* genes [40], and *AMA1* appears to be expressed at least in the merozoite stage of the parasite as shown by the sequencing of EST/cDNA from *Theileria annulata* merozoites (http://old.genedb.org/genedb/Search?submit=Search+for&name=TA02980&organism=annulata&desc=yes&wildcard=yes). This raises the intriguing hypothesis that AMA1 and the RONs might constitute a conserved molecular kit unrelated to TJ formation *per se*, possibly involved in a basic interaction with the host cell such as attachment–detachment. Are AMA1 and the RONs present at the *Theileria*–erythrocyte/lymphocyte TJ-free interface, and do they form a complex in *Theileria* extracts?

What is the role of the AMA1–RON complex?

The direct functional evidence gathered from the *Plasmodium* and *Toxoplasma* mutants thus suggests a model in which AMA1 and RON play, at least in part, independent roles and the AMA1–RON complex does not structure the TJ. What role could the conserved complex play in this model? One possibility is that it still plays an important role in TJ function, in which case the undetectable amounts of AMA1 in the invasive knockdown *Toxoplasma* tachyzoites and *Plasmodium* sporozoites would indeed be crucial for their normal invasion phenotypes. For example, minute amounts of AMA1 might be sufficient if AMA1 is involved in transient interactions with RON2 during TJ assembly. Alternatively, small amounts of AMA1 would also be compatible with a signaling role: AMA1 binding to RON2 might sense a formed TJ and activate subsequent steps of the invasion process, such as rhoptry secretion as previously suggested [16,35]. In such cases, AMA1 would have two functions, one in zoite adhesion and a second, RON-dependent, at the TJ. This case would predict that *Plasmodium* sporozoites with an *AMA1* knockout would be noninvasive if the complex has an essential role, or less invasive if the complex has a facilitator role sufficient for its conservation.

Another possibility is that the complex is not involved in TJ formation or zoite traction, but the AMA1–RON association is important for a downstream event, for example cleavage of surface AMA1 at the TJ. Although a function of the TJ in sieving host cell transmembrane proteins during