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Invasion factors of apicomplexan parasites: essential or redundant?

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Apicomplexa are obligate intracellular parasites that cause several human and veterinary diseases worldwide. In contrast to most intracellular pathogens these protozoans are believed to invade a rather passive host cell in a process, that is, tightly linked to the ability of the parasites to move by gliding motility. Indeed specific inhibitors against components of the gliding machinery and the analysis of knockdown mutants demonstrate a linkage of gliding motility and invasion. Intriguingly, new data show that it is possible to block gliding motility, while host cell invasion still occurs. This suggests that either the current models established for host cell invasion need to be critically revised or that alternative, motor independent mechanisms are in place including a more active role of the host cell that can complement a missing actin–myosin-system. Here we discuss some of the discrepancies that need to be addressed for a better understanding of invasion.

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Introduction

Apicomplexa are obligate intracellular parasites and therefore the invasion of the host cell is an essential step during their life cycle. While most pathogens rely on the modulation of host cell factors to trigger their own uptake via endocytosis or phagocytosis, most apicomplexan parasites penetrate their host cell in the absence of any visible membrane ruffling in an active process [1] that seems to be tightly linked to the parasites ability to move by gliding motility [2].

Host cell invasion is a stepwise process that can be roughly defined in four steps. First, host cell approach; second, host cell recognition; third, formation of a tight junction with the host cell and fourth, host cell penetration. During these steps the ability of the parasite to move by gliding motility plays a crucial role in host cell approach and possibly host cell penetration [2]. Gliding motility requires the action of the Myosin A (MyoA)-motor complex, termed the glideosome [3], parasite actin [4] and micronemal transmembrane proteins of the TRAP family [5,6] that interact with actin via the glycolytic enzyme aldolase [7]. The sequential secretion of micronemes and rhoptries [8•,9] leads to the formation of a tight junction between the parasite and the host cell and it is believed that the gliding machinery provides the necessary force during the penetration process through the junction (Figure 1; for recent reviews see [2,10–12]). Surprisingly, recent reverse genetic studies demonstrated that core components (including actin, MyoA, TRAP-family proteins and AMA1) of the invasion machinery can be removed without blocking host cell penetration [13••,14••]. This study leads to two interpretations. First, all apicomplexan parasites use a single entry mechanism and hence the current invasion model is wrong and needs to be replaced by a new model. Second, the current model is overall valid but an additional, motor independent invasion mechanism is at work that facilitates host cell invasion in KO mutants of the glideosome. The latter could also suggest that significant differences exist in the invasion mechanisms of different species and stages of apicomplexans.

Here we re-evaluate previous key-findings that support the current invasion model and discuss alternative mechanisms apicomplexan parasites might have at their disposal in order to invade a host cell.

Gliding motility is not coupled to invasion

Gliding motility is not restricted to apicomplexan parasites, but is also present in free living protozoans. For example, an almost identical form of gliding motility can be found in other members of the chromealveolates, such as free-living diatoms that use their actin–myosin motor to move in a substrate dependent manner [15,16]. Gregarines represent a group of early emerging apicomplexans that parasitise invertebrates and urochordates. They approach the host cell by gliding motility [17] but do not invade. Instead they tightly attach and feed on the host cell with their apical pole partially integrated [18•]. For the acquisition of nutrients the gregarines evolved sophisticated adaptations of their

apical complex that include unique secretory organelles. Similarly, free living relatives of apicomplexan parasites, such as the colpodellids feed by myzozytosis (cellular vampirism), which requires the establishment of a hole in the host cell membrane [19,20]. Intriguingly, apicomplexans such as *Plasmodium* and *Toxoplasma* also secrete the content of their rhoptries to establish a ring-like structure through which they invade [10]. This raises the question if myzozytosis and host cell penetration are based on similar molecular mechanisms that do not require gliding motility.

Theileria sporozoites are non-motile and do not show the typical apical complex with micronemes being absent. Unlike its relatives *Theileria* can enter the host cell in any orientation, independent of parasite and host cell actin [21]. A zippering mechanism has been proposed that involves the formation of multiple, tight interactions between the parasite and the host cell [22]. Whether this mechanism is homologous to a zoite that invades in an apical orientation, if the zippering mechanism can provide the necessary force for rapid invasion or if other force-generating mechanisms are involved in host cell penetration is not clear.

Cryptosporidium invades the host cell but remains extracytoplasmatic and is capable of extensive actin remodelling within the host cell [23]. Furthermore, *Cryptosporidium* recruits host cell sugar transporters and aquaporins to the attachment site to generate a diffusion gradient that allows the rapid generation of protrusions [24]. Interestingly, recent studies demonstrate that other apicomplexans such as *Toxoplasma gondii* and *Plasmodium berghei* are capable of modulating the actin cytoskeleton of the host cell, which play an important role during invasion [25,26•] and it remains to be seen which host cell factors can be recruited to the attachment zone to facilitate invasion by these parasites.

In summary, these observations hint at the existence of distinct invasion mechanism in apicomplexans (Figure 1) and it raises the question of whether certain apicomplexans use the gliding machinery to generate a force at the tight junction that allows them to gain access to the host cell. If so, the question is whether this mechanism is used exclusively or in addition to others.

Gliding motility a driving force for invasion?

Several lines of evidence support a model, where at least in case of *T. gondii* and *Plasmodium* spp. the gliding machinery powers host cell penetration. However, recent advances in reverse genetics allowed the detailed re-dissection of this machinery and demonstrated that key-invasion factors such as AMA1 [14••], MIC2, MyoA or actin [13••] can be removed without abolishing host cell invasion. Therefore a re-evaluation of previous findings is required.

Inhibitor studies

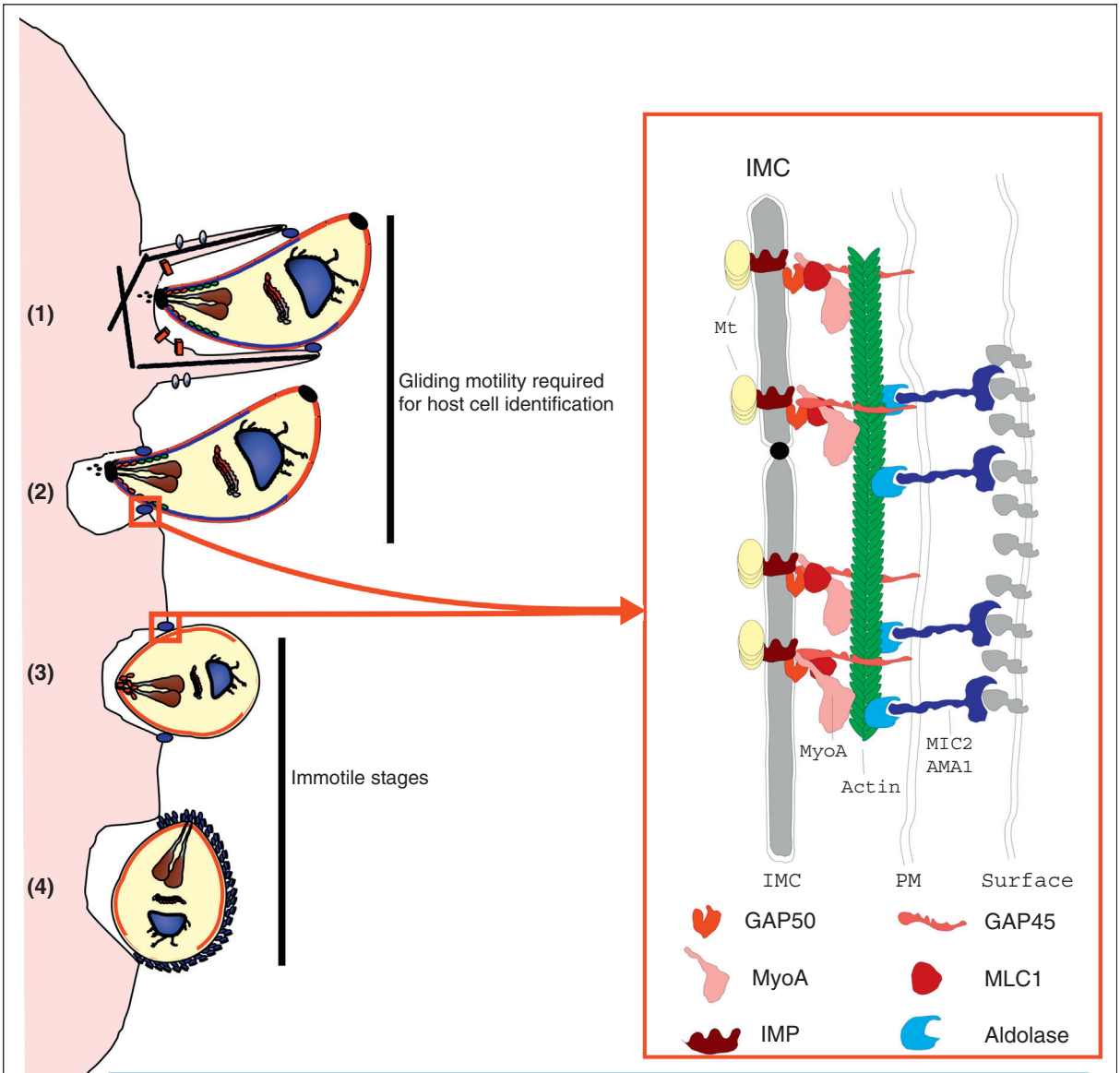
The rapid entry of apicomplexans into the host cell has puzzled researchers for decades and several potential uptake mechanisms, including zippering mechanisms or chemical induction of endocytotic events have been discussed [27,28]. Cytosolic components of the host cell, such as ATP or magnesium ions are important for host cell invasion by *Plasmodium* merozoites [29–34] and *Toxoplasma* [35,36], highlighting the importance of the host cell environment for invasion. While these studies reported only reductions in host cell invasion, a complete block can be achieved by treating the host cell with the actin destabilising drug cytochalasin D (CD). Intriguingly, three independent studies used CD to dissect the role of parasite and host cell actin during invasion in *T. gondii* reached different conclusions [37,25,38]. The first study by Ryning and Remington [37] carefully compared the uptake of *T. gondii* and heat-killed *Candida* by phagocytic and non-phagocytic cells. Although the authors could not exclude that CD also inhibits actin of the parasite, they demonstrate that preincubation of parasites for 30 min with 10 μg CD (=19.7 μM), followed by dilution to a final concentration of 0.39 μM did not significantly affect parasite invasion and conclude:

"*The results of this study suggest that T. gondii acts on the nonphagocytic cell in some undefined way, inducing phagocytosis rather than utilizing the cells merely as a passive agent during the entry process. If this were not the case, it would be unlikely that the inhibition of entry of T. gondii and its reversibility after withdrawal of CD from the medium to which the cells were exposed would exactly parallel inhibition and recovery of T. gondii entry into PM, as well as phagocytosis of heat-killed Candida by macrophages. Our studies do not preclude the concept that T. gondii actively gains entry into nonphagocytic cells without active participation by the host cell; however, we consider this a less likely mechanism.*" [37].

With the onset of genetic manipulation, the role of parasite and host cell actin was re-addressed using a combination of epithelial cell and *T. gondii* mutants resistant to CD [39]. Here, a low CD concentration of 0.2 μM was chosen to demonstrate that CD resistant parasites invade better then wild type parasites and it was concluded that invasion exclusively depends on parasite actin. Curiously, one of the mutant parasite lines resistant to CD was found *not* to have mutations in actin [39], leading to the question what alternative mechanisms the parasite has at its disposal to invade in presence of low doses of CD.

Strangely, a recent study [25] reported slightly higher concentrations of CD (0.5 μM) to be sufficient to completely block invasion by the CD resistant parasites [25]. Furthermore this study shows that host cell actin accumulates at the point of entry, in a mechanism that might be similar to *Cryptosporidium* [23]. Therefore, three

Figure 1



Gene	Knockdown	Gliding	Invasion	Knockout	Essential	Invasion
MyoA	Meissner et al., 2002	Blocked	Not blocked	Andenmatten et al., 2013	No	Not blocked
MIC2	Huynh et al., 2005	Circular	Not blocked	Andenmatten et al., 2013	No	Not blocked
Act1	No			Andenmatten et al., 2013	Yes	Not blocked
GAP45	Frenal et al., 2010	Blocked	Not blocked	Egarter et al., unpublished	Yes	Not blocked
MLC1	No			Egarter et al., unpublished	Yes	Not blocked
Profilin	Plattner et al., 2008	Blocked	Not blocked	No		
ADF	Mehta et al., 2011	Blocked	Not blocked	No		

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Left: Different modes of host cell invasion by different apicomplexans. (1) Motile zoite, that is, capable to significantly modify the cytoskeleton of the host cell and to recruit different host cell surface proteins to the attachment zone, as described for *C. parvum*, *T. gondii* and *Plasmodium* sporozoites. (2) Motile zoite that invades the host cell using its own actin–myosin-motor to invade a passive host cell, as described for *T. gondii* and *Plasmodium*

independent studies come to different conclusions regarding the role of host cell and parasite actin during invasion hinting that both may be involved. Furthermore, the demonstration that depletion of actin in *T. gondii* does not abrogate host cell invasion strongly suggests that invasion does not exclusively depend on parasite actin [13••].

The myosin ATPase inhibitor butanedione monoxime (BDM) has been used to demonstrate a role of myosins during the penetration of the host cell [40,41]. However, BDM is a relatively non-specific inhibitor and affects multiple cellular processes, such as ionic current flow [42,43]. Indeed, one study suggests that the unconventional myosins of apicomplexans are not specifically inhibited by BDM [44]. A similar problem as for CD might well be encountered with Latrunculin, which has been claimed to inhibit actin driven processes in *Toxoplasma* and *Plasmodium falciparum* but strikingly did not inhibit the extremely cytochalasin and jasplakinolide sensitive gliding motility of *P. berghei* sporozoites [45].

The confusion created by using different inhibitor concentrations and different parasite lines in different experiments are reminiscent of a previous study using a specific inhibitor of falcipain-1, a *P. falciparum* cystein protease, suggesting that it was essential for host cell invasion by merozoites [46]. Subsequently a genetic deletion of falcipain-1 showed that the enzyme plays no role in the erythrocytic cycle [47]. We thus suggest being more prudent when using inhibitors, that is, to perform assays over a wide range of concentrations and ideally to verify the supposed specific effect with biochemically purified protein as well as with gene deletion.

Confusing evidences from reverse genetics

Since gene deletion is often not straightforward to achieve, in *T. gondii* a tetracycline-dependent knockdown system was central for the functional dissection of the key-components of the gliding machinery [3,6,9,48,49•,50,51]. Intriguingly, *none* of the knockdowns showed the expected total block in host cell invasion, although a complete block of gliding was described in several cases (Figure 1). Invasion rates between 10% and 25% [6,9,48,49•,50,51,52•] were reported and none of these studies analysed the speed of host cell penetration (which should be significantly slower) in detail. Together these studies demonstrate that knockdown of motor components although sufficient to block gliding motility on glass slides does not result in a block of host cell invasion leading to the question how much force needs to be generated at the tight junction by this motor to facilitate host cell penetration. In this respect the phenotype of one mutant is very intriguing: a knockdown for GAP45. In the absence of GAP45 the remaining components of the gliding machinery (MyoA and MLC1) are relocated to the cytosol of the parasite leading to a block of motility [53••]. Furthermore the integrity of the IMC, which is a prerequisite to stabilise the glideosome [54,55] is lost. Yet, this mutant invades with an overall invasion rate of 20% [53••]. How is this possible, if not only the MyoA-motor itself, but the whole platform for other, possibly redundant motors is missing?

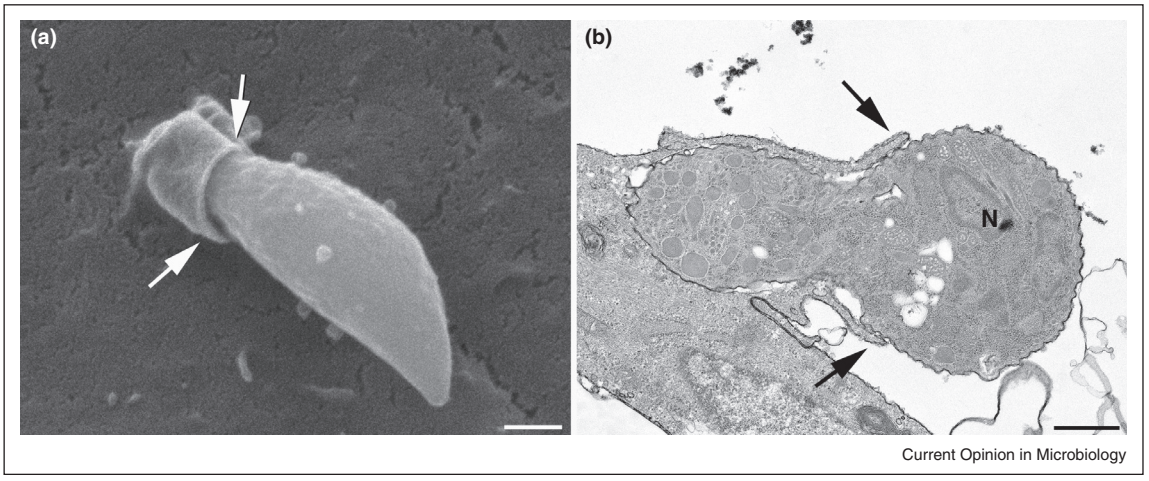
While the uncoupling of gliding and host cell invasion can be attributed to background expression levels of the gene of interest (GOI) in the respective knockdowns, the essentiality of the gliding machinery for host cell invasion has not been demonstrated. Rather it appears that even low background expression level is sufficient to allow for host cell invasion.

To address this obvious discrepancy, a ligand controlled site-specific recombination system based on dimerisable Cre-recombinase (DiCre) was adapted to *T. gondii* [13••]. This allowed the complete removal of several genes of interest (GOI) and the generation of conditional knockouts for MIC2, AMA1, MyoA, MLC1, GAP45 and Act1 in *T. gondii* ([13••], Figure 1; MM, unpublished results). Clonal knockouts from the induced population could be isolated for several factors previously described as essential, including MIC2, AMA1 and MyoA. This clearly demonstrates that they are not essential for *in vitro* growth and that parasites are capable to invade the host cell in the absence of the actin–myosin-system ([13••]; MM, unpublished). While these reverse genetic data demonstrate the existence of an actin–MyoA–AMA1/MIC2 independent invasion pathway in *T. gondii* tachyzoites it cannot yet be ruled out that several redundant mechanisms for host cell invasion exist in apicomplexan parasites and that different species prefer one over the other.

Or is it possible that our model for gliding motility and invasion needs to be revised? Currently the linear motor model predicts that myosin and actin provide the force necessary for gliding. However, it is also plausible that the primary function of myosin and actin is the definition (formation and/or release) of attachment sites [56] that provide directionality of the movement. The force itself could be easily generated by other means, for example, through hydrodynamic forces generated by cytosolic fluid dynamics that have been implicated in rapidly moving

sporozoites. (3) Immotile zoite that invades the host cell using its own actin–myosin-motor to invade a passive host cell, as described for *Plasmodium* merozoites. (4) Immotile zoite that invades the host cell using a zippering mechanism to invade the host cell, as described for *Theileria* merozoites. Right: A simplified model of the actin–myosin-system beneath the surface of the parasite. Some key-factors that have been studied using knockdown or knockout approaches are indicated. The model is not drawn to scale. The table provides a summary of knockdown and knockout studies on some of the key components for the gliding machinery.

Figure 2



Formation of a collar-like structure during invasion of the host cell by *T. gondii*. Scanning **(a)** and transmission **(b)** electron micrographs of tachyzoites of *Toxoplasma gondii* invading culture human umbilical endothelial cells. The apical end of the parasite is orientated towards the epithelial cell but there also appears to be the development of a protrusion the host cell plasmalemma to form collar around the invading parasite. N — nucleus. Bars represent 1 μm.

cells, such as keratinocytes [57]. In this case one would predict that myosin and actin are required during gliding motility to determine the directionality of the movement. However, once the parasite is attached to the surface of the host cell, the direction is defined by the tight junction and consequently myosin and actin function is not required.

Although actin, myosins and micronemal proteins appear to have an important function in steps upstream of host cell penetration, recent findings and a critical review of the literature do not provide a conclusive argument for their essential role during host cell penetration. Therefore it is also necessary to re-investigate the role of the host cell during this process.

The role of the host cell in parasite penetration

Apicomplexan parasites are capable of significantly modifying and hijacking host cellular functions [58], although our current view is that this mainly occurs in order to secure parasite survival after invasion. Several studies point out that apicomplexan invasion does at least to a certain extent depend on cytosolic host cell factors, such as actin, tubulin, ATP, magnesium ions, aquaporins and sugar transporters [23,24,59,60•]. While these studies cannot demonstrate a complete block in host cell invasion by modulating these factors, it is possible that a parasite modulates multiple parameters in order to gain rapid access to the host cell and clearly demonstrates that the host cell is not as passive as previously believed. Indeed, it is likely that different parasites and different parasite stages modulate different cellular parameters and use different proteins in different ways, thus limiting the theory of a uniform invasion mechanism.

While triggering phagocytosis or endocytosis appears to be an unlikely mechanism, due to the rapid nature of the penetration (~30 s), it has been shown that host cell actin and tubulin are recruited to the attachment site and that their modulation contributes to parasite invasion [25,61]. Indeed ultrastructural evidences show that upon attachment a collar like structure can form between *T. gondii* and the non-phagocytic host cell (Figure 2; DF, unpublished). Since invasion of parasites without these protrusions can also be readily observed, the formation of this collar might represent an alternative invasion mechanism, where the host cell plays an active role during the penetration process.

However, the question still remains: how can parasites penetrate the host cell in the absence of their gliding machinery? Clearly more work needs to be done as the field suddenly seems to be once again 'wide open'.

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