Invasion factors are coupled to key signalling events leading to the establishment of infection in apicomplexan parasites

SANTOS, Joana M, SOLDATI-FAVRE, Dominique

Abstract

Invasion of host cells by apicomplexan parasites is initiated when specialized secretory organelles called micronemes discharge protein complexes onto the parasite surface in response to a rise in parasite intracellular calcium levels. The microneme proteins establish interactions with host cell receptors, engaging the parasite with the host cell surface, and signal for the immediate exocytosis of another set of secretory organelles named the rhoptries. The rhoptry proteins reprogram the invaded host cell and participate in the formation of the parasitophorous vacuole in which the intracellular parasite resides and replicates. Disengagement of the invading parasite from the host cell receptors involves the action of at least one parasite plasma membrane rhomboid protease, which is concomitantly implicated in a checkpoint that signals the parasite to switch from an invasive to a replicative mode.
Microreview

Invasion factors are coupled to key signalling events leading to the establishment of infection in apicomplexan parasites

Joana M. Santos and Dominique Soldati-Favre*
Department of Microbiology, Faculty of Medicine, University of Geneva, 1 rue-Michel Servet, 1211 Geneva 4, Switzerland.

Summary

Invasion of host cells by apicomplexan parasites is initiated when specialized secretory organelles called micronemes discharge protein complexes onto the parasite surface in response to a rise in parasite intracellular calcium levels. The microneme proteins establish interactions with host cell receptors, engaging the parasite with the host cell surface, and signal for the immediate exocytosis of another set of secretory organelles named the rhoptries. The rhoptry proteins reprogram the invaded host cell and participate in the formation of the parasitophorous vacuole in which the intracellular parasite resides and replicates. Disengagement of the invading parasite from the host cell receptors involves the action of at least one parasite plasma membrane rhomboid protease, which is concomitantly implicated in a checkpoint that signals the parasite to switch from an invasive to a replicative mode.

Introduction

The phylum Apicomplexa groups unicellular eukaryotic pathogens, including Toxoplasma gondii and Plasmodium, the causative agents of toxoplasmosis and malaria respectively. Host cell invasion by these parasites is an active process, which critically relies on the parasite actomyosin system (glideosome) and the sequential secretion of proteins from two types of specialized apical organelles, micronemes and rhoptries (Carruthers and Sibley, 1997). The microneme proteins (MICs) are discharged onto the parasite surface upon the release of calcium from intracellular stores (Carruthers and Sibley, 1999; Lovett et al., 2002; Lovett and Sibley, 2003) and form tight interactions with host cell receptors. Assembly of these complexes is essential for glideosome function (Huynh et al., 2003) and formation of the moving junction (MJ), an electron dense constriction produced at the point of apposition between the parasite and the host cell membranes (Aikawa et al., 1978; Michel et al., 1980). During invasion, the MIC-receptors complexes are re-distributed towards the posterior end of the parasite powered by the glideosome, and as a result the parasite is propelled into the host cell. Concomitantly, migration of the MJ results in the formation of a specialized vacuole called the parasitophorous vacuole (PV) (Mordue et al., 1999). Recent studies have indicated that the binding of the MICs to host cell receptors triggers exocytosis of the rhoptry components (Singh et al., 2010), which in turn contribute to the formation of the MJ, participate in formation of the PV and modify the invaded host cell [reviewed in (Boothroyd and Dubremetz, 2008)]. At the end of the invasion process, the MIC adhesins are released from the parasite surface by proteolytic cleavage and there is sealing of the PV, resulting in parasite disengagement from the host cell membrane. Once secluded inside the PV, the parasite can initiate replication [reviewed in (Carruthers and Boothroyd, 2007)]. Most intracellular stages of the life cycle 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 invasive at the time of host cell egress. In this review, we will summarize the most recent advances in the study of the mechanisms that lead to the establishment of infection by Plasmodium and Toxoplasma and pinpoint the similarities and differences between the two parasites.

MICs assemble into multi-protein complexes

The majority of MICs identified in T. gondii form complexes, which preassemble in the endoplasmic reticulum.
prior to transit to the micronemes. Four complexes have been functionally characterized. Micronemal protein 2 (TgMIC2), a member of the conserved thrombospondin-related adhesive protein family, is found in a heterohexamer complex with MIC2-associated protein (TgM2AP) and plays a fundamental role in gliding motility, host cell attachment and invasion (Huynh and Carruthers, 2006). TgMIC6 forms a complex with two adhesins (TgMIC1 and TgMIC4) and contributes to invasion in vitro and virulence in vivo (Cerede et al., 2005; Blumenschein et al., 2007; Sawmynaden et al., 2008). TgMIC8 assembles with a lectin (TgMIC3) and is essential for rhoptry secretion and invasion (Kessler et al., 2008). The fourth complex is unusual in that it assembles into its final, functional form only at the MJ following exocytosis (Alexander et al., 2005; Straub et al., 2009; Besteiro et al., 2009), bringing together the MIC apical membrane antigen 1 (TgAMA1) and several preassembled rhoptry neck proteins (RONs) (TgRON2-RON4-RON5-RON8). Parasites deficient in TgAMA1 still attach to host cells but are defective in rhoptry secretion and hence fail to form the MJ and invade host cells (Mital et al., 2005). Unlike the complexes mentioned above that are specific to T. gondii and Neospora caninum, the AMA1-RONs complex appears to be conserved across the phylum, with the exception of Cryptosporidium (Cao et al., 2009; Collins et al., 2009; Straub et al., 2009).

The transmembrane MICs present in each complex – TgMIC2, TgMIC6, TgMIC8 and TgAMA1 – (Fig. 1A) all portray a modular structure comprising an ectodomain, a membrane-spanning domain and a short cytoplasmic tail. While the ectodomains establish connections with host receptors and hence engage the parasite with the host cell surface (Fig. 1B), the C-terminal domains can sometimes escort the complex to the micronemes and may associate with the glideosome via binding to aldolase. While TgMIC2, TgMIC6 and TgAMA1 have all been shown to bind to aldolase, at least in vitro (Jewett and Sibley, 2003; Zheng et al., 2009; Sheiner et al., 2010), only TgMIC2 and TgMIC6 are proven to function as escorters (Di Cristina et al., 2000; Reiss et al., 2001; Opitz et al., 2002).

**Signalling events leading to microneme secretion**

Regulated secretion of the micronemes depends on signal transduction cascades involving calcium as a key intracellular messenger (Carruthers et al., 1999a; Chen et al., 2004). Organelle discharge can be induced in extracellular parasites by pharmacological agents such as ethanol and calcium ionophores (Carruthers and Sibley, 1999; Carruthers et al., 1999b; Lovett et al., 2002; Nagamune et al., 2007) and it is inhibited by treatment with intracellular calcium chelators (Carruthers et al., 1999a).

Elevation of the parasite intracellular free calcium levels occurs upon exocytosis of ER calcium stores after both cADPR and IP3 stimulus (Carruthers et al., 1999b; Lovett et al., 2002; Lovett and Sibley, 2003; Chini et al., 2005; Nagamune and Sibley, 2006; Nagamune et al., 2007). Once penetration of the host cell is completed, the intracellular calcium levels drop rapidly as a result of negative feedback, and secretion events in intracellular parasites are undetectable (Carruthers et al., 1999a; Lovett and Sibley, 2003; Singh et al., 2010).

Egress from host cells also involves microneme discharge and the concerted action of the MICs in Toxoplasma (Kafsack et al., 2009). Disruption by homologous recombination of the gene coding for TgMIC perforin-like protein 1 (TgPLP1) leads to a marked impairment in calcium ionophore-induced egress. This defect is not caused by interference with parasite motility but due to the lack of rupture of the PV membrane. Interestingly, TgPLP1 can act in trans as tgplp1ko parasites are able to egress from host cells co-infected with wild-type parasites.

Calcium-mediated signalling pathways in T. gondii and Plasmodium have been consistently linked to the action of calcium-dependent protein kinases (CDPKs) [reviewed in (Nagamune et al., 2008)], which are otherwise only found in plants. Phylogenetic analysis indicates that T. gondii and P. falciparum encode 11 and 8 CDPK-like genes, respectively (Lourido et al., 2010), and crystal structure studies revealed a striking conservation between the plant and apicomplexan CDPKs (Wernimont et al., 2010). The CDPKs possess a Ser/Thr kinase domain highly homologous to that of vertebrate calmodulin-dependent kinases (CaMKs) fused to four EF-hand domains (Nagamune and Sibley, 2006). Enzymatic activation depends on binding of calcium to the calmodulin-like domain, which induces a dramatic structural rearrangement in the kinase domain, releasing it from an inactive conformation (Harper and Harmon, 2005; Wernimont et al., 2010).

A recent description of the conditional knockdown of TgCDPK1 has validated this kinase as a key mediator of the signalling cascade leading to microneme secretion (Lourido et al., 2010). Because of a severe defect in microneme secretion, tgcdkp1iko parasites fail to attach, invade or egress host cells. Moreover, the fact that these parasites are unable to egress when treated with calcium ionophores indicates that TgCDPK1 acts downstream of the calcium signal that regulates microneme exocytosis (Lourido et al., 2010). TgCDPK1 substrates remain unknown for the moment. Microneme exocytosis is also regulated by the cyclic guanosine 3′, 5′-cyclic monophosphate (cGMP) pathway. Inhibition of cGMP-dependent protein kinase (PKG) with a selective inhibitor impairs P. berghei ookinete gliding motility (Moon et al., 2009) and T. gondii attachment, invasion and gliding (Wiersma et al., 2010).
Intriguingly, PKG activity is independent of CDPK1 function in T. gondii (Wiersma et al., 2004; Lourido et al., 2010) but in P. berghei the cGMP- and calcium-dependent pathways of microneme exocytosis converge. Indeed, deletion of a cGMP-degrading enzyme (phosphodiesterase) in the strain deleted for PbCDPK3, which is affected in ookinete movement (Siden-Kiamos et al., 2006), restored a normal motility phenotype (Moon et al., 2009). PKG has also been implicated in secretion of Plasmodium exonemes (Dvorin et al., 2010a), which discharges a protease required for parasite egress from the host erythrocyte (Yeo et al., 2007). PICDPK5 is also involved in egress but appears to act downstream in the signalling cascade (Dvorin et al., 2010a).
adenosine 3′, 5′-cyclic monophosphate (cAMP) pathway is also involved in organelle exocytosis in Plasmodium sporozoites and merozoites by a mechanism dependent on protein kinase A (PKA) (Beraldo et al., 2005; Ono et al., 2008; Leykauf et al., 2010). The mobilization of calcium induced by cAMP is also seen upon stimulation of inositol 1,4,5-triphosphate production via the phospholipase C (PLC) pathway, suggesting that the cAMP and PLC pathways are linked (Beraldo et al., 2005).

The RONs and rhoptry bulb proteins: two different families of rhoptry proteins

The rhoptry organelles store lipids and proteins that can be found either at the neck (RONs) or at the bulb of the organelle (rhoptry bulb proteins, ROPs) (Fig. 1A). The ROPs and RONs can be distinguished not only in terms of their localization in the organelle but also by the timing of discharge and their ultimate destination and function.

The RONs have been so associated with the formation of the MJ (Fig. 1) together with AMA1, both in T. gondii tachyzoites (Alexander et al., 2005) and in P. falciparum merozoites (Cao et al., 2009; Collins et al., 2009). In Toxoplasma the MJ complex is composed of TgRON2, TgRON4, TgRON5 and TgRON8 (Alexander et al., 2005; Besteiro et al., 2009; Collins et al., 2009). In Plasmodium, RON8 is absent and PIRON2, PIRON4 and PIRON5 form a pre-complex in the rhoptries, which is then delivered onto the parasite’s surface (Collins et al., 2009) where it binds to AMA1 via RON2 (Alexander et al., 2005; Besteiro et al., 2009; Collins et al., 2009; Tyler and Boothroyd, 2011). Only a minority of AMA1 present on the parasite surface associates with the RONs at the MJ (Alexander et al., 2005; Collins et al., 2009; Tyler and Boothroyd, 2011). The exact function of the MJ complex is still a matter of debate but it is known to be required only after parasite reorientation and establishment of the initial tight junction (Richard et al., 2010). In T. gondii the RONs complex is targeted to the host cell membrane during invasion and one hypothesis is that it effectively serves as the parasite’s own receptor at the host cell surface, perhaps contributing to the ability of T. gondii to invade a wide range of cell types (Besteiro et al., 2009). It should be noted however that Plasmodium, which is capable of invading a much more restricted host cell repertoire, also possesses the AMA1-RONs complex (Cao et al., 2009; Collins et al., 2009).

Unlike the known RONs, which are restricted to the MJ, the ROPs are delivered either to the PV or into the host cell (Fig. 1C) (Carruthers and Sibley, 1997; Alexander et al., 2005). The largest family of Toxoplasma ROP genes encodes at least 44 kinases and pseudokinases, including 16 predicted to be active (Peixoto et al., 2010), and some of them have been shown to be involved in post-invasion manipulation of host cell signalling [reviewed in (Pollard et al., 2009)]. The rhoptries also release phosphatases, proteases and other proteins of unknown function [reviewed in (Boothroyd and Dubremetz, 2008)]. Additionally the dense granules also contribute to the discharge of proteins capable of acting as effectors and subverting host functions. A recent example is TgGRA15, which activates the NF-κB pathway (Rosowski et al., 2011).

Signalling for rhoptry secretion is coupled to MIC function

In Plasmodium, recent studies have indicated that signalling for rhoptry secretion is mediated by binding of the parasite micronemal erythrocyte binding antigen-175 (PfEBA-175) or -140 (PfEBA-140) to their respective host receptors (Singh et al., 2010) (Fig. 1C). The current model for rhoptry secretion advocates that adhesin-receptor binding restores the basal calcium levels, which were elevated to induce microneme discharge, through a signal transmitted by the C-terminal tails of the PfEBA family members (Singh et al., 2010). The exact signal transduction mechanism remains to be discovered.

In Toxoplasma both TgAMA1 (Mital et al., 2005) and TgMIC8 (Kessler et al., 2008) are involved in rhoptry secretion. In parasites conditionally depleted in TgAMA1 there is still secretion of the TgRONs (Alexander et al., 2005) but release of the TgROPs is impaired (Mital et al., 2005). In contrast, a complete abrogation of rhoptry secretion is observed in the absence of TgMIC8 (Kessler et al., 2008). Curiously, inhibition of T. gondii phospholipase A2 blocks rhoptry secretion in a similar manner to TgMIC8 absence (Ravindran et al., 2009). The exact signalling mechanism remains unknown but it is plausible that the binding of TgMIC8 to an unknown host cell receptor signals for secretion of the TgRONs and, subsequently TgAMA1 association with the TgRON complex triggers TgROP secretion. Such a model implies physical discontinuity between the necks and the bulbs of the rhoptries, which remains to be assessed, and interference with the TgAMA1 role during invasion does not impair TgROP injection into the host cell (Tyler and Boothroyd, 2011).

MICs are proteolytically processed during invasion

The TgMICs undergo extensive proteolytic remodelling. The first cleavage event removes the signal peptide and subsequently a series of other cleavages occur along the secretory pathway (Dowse and Soldati, 2004; Carruthers, 2006). Some TgMICs also contain propeptides that are removed by a cathepsin-like protease (Parussini et al., 2010). Further proteolytic processing events occur post exocytosis at the parasite surface involving three distinct
enzymatic activities referred to as micronemal protein proteases -1, -2 and -3 (MPP1, MPP2 and MPP3) (Carruthers et al., 2000; Zhou et al., 2004). While MPP2 and MPP3 perform the so-called surface trimming, mediated by the micronemal subtilisin-like protease TgSUB1 (Lagal et al., 2010), MPP1 mediates shedding of the transmembrane TgMICs from the parasite surface (Fig. 1C).

MPP1 is a constitutively active, resident plasma membrane protease (Opitz et al., 2002), which activity appears to be controlled by compartmentalization of the substrates in the micronemes. MPP1 is an essential and likely conserved proteolytic activity across the Apicomplexa phylum (Opitz et al., 2002) but its biological function remains to be elucidated. It has been suggested that MPP1: (i) sheds MICs from the parasite surface so that they cannot be targeted by neutralizing antibodies (Carruthers and Boothroyd, 2007); (ii) creates a gradient of MICs from the anterior to the posterior end of the parasite, contributing to parasite reorientation (Bugarulisks et al., 2010); (iii) disengages the parasite from the host cell at the end of the invasion process; or (iv) assures proper function of the glideosome by releasing the MIC-host cell receptor complexes during movement (Carruthers and Boothroyd, 2007).

MPP1 was shown to cleave TgMIC2, TgMIC6, TgMIC12 and TgAMA1 within their transmembrane domain (Opitz et al., 2002; Brossier et al., 2003; Urban and Freeman, 2003; Zhou et al., 2004; Howell et al., 2005) at amino acid motifs reminiscent of those targeted by rhomboid-like serine proteases (Dowse et al., 2005; Amaranth and Rawson, 2009; Strisovsky et al., 2009). While T. gondii expresses two rhomboid-like proteases (TgROM4 and TgROM5) at the plasma membrane (Fig. 1A), the malaria parasite only possesses a ROM4 homologue (Brossier et al., 2005; Dowse et al., 2005; O’Donnell et al., 2006; Sheiner et al., 2008). Cell-based cleavage assays have validated TgROM5 (Brossier et al., 2005; Dowse et al., 2005) and PfROM4 (Baker et al., 2006; O’Donnell et al., 2006) as active enzymes with broad substrate specificity (Baker et al., 2006) but TgROM4 was either inactive or not expressed in this context (Brossier et al., 2005; Dowse et al., 2005). However, the recent conditional knockout of TgROM4 demonstrated that this protease is active on the parasite surface and likely cleaves TgMIC2, TgAMA1 and TgMIC8 (Buguliskis et al., 2010). TgROM4 depletion caused ~50% reduction in invasion and did not affect TgMIC6 shedding, despite the sequence similarity between TgMIC2 and TgMIC6 rhomboid cleavage motifs. These results suggest that the MPP1 activity in Toxoplasma may be mediated by two rhomboid proteases with distinct substrate specificities (Buguliskis et al., 2010). A new model for MPP1 activity can thus be envisioned in which TgROM4 and TgROM5 both perform important and not necessarily overlapping functions. As TgROM4 is evenly distributed over the parasite surface, it might contribute to the creation of the gradient of adhesins and to glideosome function (Fig. 1C), as recapitulated by the phenotype of the tgron4/ko parasites (Buguliskis et al., 2010). At the end of penetration, TgROM5, which preferentially localizes to the posterior end of the parasite, might execute the final pinching activity during closure of the PV and disengagement of the parasite from the host cell (Fig. 1D) (Brossier et al., 2005; Dowse et al., 2005). Validation of TgROM5 as mediator of some aspects of MPP1 activity awaits further investigation. In Plasmodium, only PfROM4 is expressed at the parasite surface (O’Donnell et al., 2006) but the parasite also possesses a subtilisin-like enzyme named Pfsub2, which acts as a surface sheddase during invasion (Howell et al., 2005).

**Coupled functions of TgROM4 and TgAMA1 in invasion and replication**

In parallel to the study of TgROM4 with a conditional knockout (Buguliskis et al., 2010), TgROM4 function was investigated by generating of transgenic parasites expressing an inducible dominant negative TgROM4 form. The protease carrying a mutation in the catalytic serine (ddROM4S-A) was predicted to sequester its substrate(s) from the active endogenous TgROM4 copy (Santos et al., 2010) (Fig. 2A). Expression of ddROM4S-A was highly deleterious to parasite survival but surprisingly, it did not inhibit invasion but severely blocked parasite replication (Santos et al., 2010). Such effect could be specifically reverted by expression of the predicted TgAMA1 rhomboid cleavage product (TgAMA1-tail), suggesting that TgROM4-mediated cleavage of TgAMA1 is not essential for invasion but switches instead the parasite from an invasive to a replicative mode (Santos et al., 2010). Rhomboid proteases have been previously associated with intercellular signal transduction events but this is the first time that a rhomboid protease has been shown to contribute to an intracellular signalling pathway [reviewed in (Urban, 2006)]. The TgAMA1-tail is also essential for invasion (Treek et al., 2009; Leykaf et al., 2010), possibly by linking the MJ complex to the glideosome via binding to aldolase (Sheiner et al., 2010), but the amino acid motif implicated in this function is not required for replication, indicating that the two functions are physically separated (Santos et al., 2010). TgROM4-mediated cleavage of TgMIC2 is essential for gliding motility (Huynh and Carruthers, 2006; Buguliskis et al., 2010), but expression of TgMIC2-tail did not complement the phenotype of ddROM4s/a parasites (Santos et al., 2010). Noteworthy, unlike TgMIC2 and the other MICs, TgAMA1 is present on the surface of invaded parasites (Howell et al., 2005). These observations are
Fig. 2. AMA1 and ROM4: coupled roles in invasion and replication.

A. Scheme representing the hypothetical mode of action of the TgROM4 dominant negative form ddROM4<sub>S-A</sub>. In the presence of Shld-1, ddROM4<sub>S-A</sub> (yellow) is stabilized and recognizes and binds to ROM4 substrates (red) but it is unable to cleave them, because of the Ala replacement at the catalytic Ser residue (orange), leading to substrate sequestration from the active endogenous TgROM4 protease (blue) and to a dominant negative effect.

B. ROM4-mediated cleavage of AMA1, at or following invasion, releases the AMA1 cytosolic C-terminal fragment (AMA1-tail), triggering parasite replication. In Toxoplasma, further cleavage of AMA1 or another ROM4 substrate are needed in order to stimulate each new cycle of replication by endodyogeny.

C. In wild-type extracellular parasites (RH), the protein levels of AMA1 (A) and ROM4 (R) are balanced and ROM4-mediated cleavage of AMA1 and other ROM4 substrates promotes host cell invasion and triggers parasite replication. In intracellular parasites, there is very little AMA1 expressed and ROM4 is in excess. In parasites expressing an inducible copy of wild-type ROM4 (ddROM4), ROM4 is in excess in both extracellular and intracellular parasites because there is expression of endogenous (black R) and inducible ROM4 (green R). In parasites expressing an inducible mutant copy of ROM4 (ddROM4<sub>S-A</sub>), expression of the mutant copy (red R) in extracellular parasites is not sufficient to abrogate cleavage of all expressed AMA1 and the parasites can efficiently invade host cells. In intracellular parasites, on the other hand, there is very little AMA1 expressed and mutant ROM4 can efficiently compete with the endogenous ROM4 for substrate binding, blocking parasite replication. In parasites knockdown for ROM4 (rom4-iKO), there is very little ROM4 expressed in both intracellular and extracellular parasites. The reduced amount of ROM4 substrates shedding impairs host cell invasion but still releases enough AMA1-tail to trigger parasite replication. In parasites knockdown for AMA1 (ama1-iKO), there is very little AMA1 expressed in both intracellular and extracellular parasites. The reduced amount of AMA1 protein expressed in extracellular parasites dramatically impairs invasion but, as expression is not completely shut down, parasites are only modestly affected in replication.

D. In Plasmodium, a single ROM4-mediated cleavage of AMA1 during invasion assures triggering of replication by schizogony.

© 2011 Blackwell Publishing Ltd, Cellular Microbiology, 13, 787–796
especially relevant in the context of parasite migration/traversal of tissues as replication should only be triggered post invasion and not during pre-invasion motility. TgAMA1 appears well suited to participate in post-invasion events along with other integrated signals generated exclusively after invasion to trigger replication and guarantee the necessary discrimination between migration and invasion.

The dominant negative effect exerted by ddROM4<sub>5,4</sub> is reversible and can be uncoupled from invasion, suggesting that each cycle of replication requires a new boost of signalling engendered by substrate cleavage (Fig. 2B). Such a phenomenon appears to be tailored to the lytic signalling engendered by substrate cleavage (Fig. 2B).

Involvement of other TgROM4 substrates. In this context, the presence in the T. gondii genome of two additional genes related to TgAMA1 and described as putative apical membrane antigens in ToxoDB (TGME49-115730 and TGME49-100130) might be of relevance.

How TgAMA1 cleavage may trigger replication remains unknown. Regulated intramembrane proteolysis is a simple and powerful strategy for signal transduction in which the substrates are membrane-anchored proteins and inactive in their membrane-tethered form. Activation occurs upon cleavage within the transmembrane domain, through release of their cytosolic or extracellular domains (Brown, 2000; Urban et al., 2002; Erez and Bibi, 2009; Freeman, 2009). It is tempting to speculate that TgROM4 is responsible for a mechanism resembling regulated intramembrane proteolysis acting on TgAMA1 that releases its cytosolic ‘tail’ to initiate a program of signal transduction (Fig. 1D), involving unknown proteins only present in intracellular parasites, that ultimately leads to expression of genes involved in parasite replication. For the moment this hypothesis is only speculative but in its support, it was recently shown that an invasion-related event triggers an immediate and sharp change in gene expression in Toxoplasma (Gaji et al., 2010).

A role for TgROM4 and TgAMA1 during replication was not observed in parasites conditionally depleted for either the protease or the MIC (Mital et al., 2005; Buguliskis et al., 2010) likely because parasites grown under repressive conditions still express background levels of TgROM4 and TgAMA1 respectively (Fig. 2C). The use of alternative experimental approaches was thus instrumental for the identification of the dual functions of TgROM4 and TgAMA1 in invasion and parasite replication. The tet-inducible system established that TgROM4 and TgAMA1 are important players during motility and invasion (Mital et al., 2005; Buguliskis et al., 2010). However, although the knockdown strategy efficiently reduced expression of TgROM4 at the surface of extracellular parasites and consequently the shedding of the TgMICs, including TgAMA1, during invasion, it failed to completely block cleavage in intracellular parasites and subsequently replication (Buguliskis et al., 2010). We hypothesize that these apparent discrepancies may be explained by the possibility that expression of ddROM4<sub>5,4</sub> is insufficient to sequester all the substrates released from the micronemes during invasion and therefore to produce a strong invasion phenotype. Expression of ddROM4<sub>5,4</sub> is also insufficient to block the first round of division following invasion, but it effectively neutralizes the limited amount of substrate delivered by intracellular parasites and thus blocks subsequent cycles of replication (Santos et al., 2010).

Although the P. falciparum AMA1-tail trans-complements the division defect imposed by expression of ddROM4<sub>5,4</sub>, it is premature to conclude that a similar mechanism dictates initiation of the replication program in malaria parasites. Unlike TgAMA1, which is exclusively cleaved by a rhomboid-like activity (Howell et al., 2005), PfAMA1 is primarily shed from the merozoite surface by juxtamembrane cleavage by the subtilisin-like protease PISUB2 (Howell et al., 2005). Indeed PIAMA1 mutations rendering it uncleavable by PIROM4 has no effect on parasite viability (C. Collins and M. Blackman, pers. comm.). As the malaria parasite replicates by schizogony, a process by which cytokinesis to produce all daughter cells occurs only once in each blood-stage cycle, it might be speculated that the cleavage of PIAMA1 by PISUB2 during invasion may be sufficient to trigger the entire replication cycle (Fig. 2D). This idea is supported by data indicating that stable parasite lines carrying PIAMA1 forms resistant to PISUB2 cleavage cannot be obtained even if these forms are functional in invasion (C. Collins and M. Blackman, pers. comm.). The cleaved form of PIAMA1 is phosphorylated at a Ser residue by PKA (Leykauf et al., 2010) and this modification is important for PIAMA1 function during invasion (Trececk et al., 2009). However, the PIAMA1-tail carried into the newly invaded ring stages appears not to be phosphorylated (Leykauf et al., 2010) raising the possibility that phosphorylation could distinguish between function during invasion and replication. Nevertheless, expression of the PIAMA1 tail mutated at the phosphorylated Ser is still able to restore replication of ddROM4<sub>5,4</sub> parasites (Santos et al., 2010).

**Conclusion**

Recent years have brought a new light into the mechanisms implicated in the establishment of infection by both
Toxoplasma and Plasmodium. While some aspects are conserved, others are specific to each parasite. The signalling pathways leading to micronemes, and most likely to rhoptries discharge, as well as function of conserved MICs appear to be conserved. Several studies have highlighted that the modular structure of the MICs allows them to perform multiple tasks. The ectodomains interact with host receptors and contribute to host cell recognition and invasion, while the cytosolic tails may carry trafficking information and link the host cell surface to the parasite glideosome or are implicated in downstream signalling events. For the moment, such dual functions have only been demonstrated for TgAMA1 and TgMIC8 in T. gondii (Mital et al., 2005; Kessler et al., 2008; Santos et al., 2010) and PFEBA-140 and PFEBA-175 in P. falciparum (Singh et al., 2010), but recent studies indicate that other MICs may mediate similar functions (Dvorin et al., 2010b).

The function of ROPs in modulating the host cell response has only been demonstrated for Toxoplasma but the presence of the RONs at the MJ is well characterized in both Toxoplasma and Plasmodium. Proteolytic processing of the MICs, while involving different players, is also largely conserved in both parasites. The new findings discussed here add a novel layer of complexity to the mechanisms leading to the establishment of infection by apicomplexan parasites and reinforce the notion of a concerted action of invasion proteins, which is tightly controlled in time and space and ultimately governs replication.

Acknowledgements

We are most grateful to Dr Michael J. Blackman stimulating discussions and for critical reading of the manuscript. JMS was previously funded by MalParTraining FP6-Marie Curie ITN (MEST-CT-2005-020492) and currently by the Swiss National Foundation (FN3100A0-116722).

References


Carruthers, V.B. (2006) Proteolysis and Toxoplasma inva-


© 2011 Blackwell Publishing Ltd, Cellular Microbiology, 13, 787–796
Cryptosporidium parvum is temperature, cytoskeleton, and intracellular calcium dependent and required for host cell invasion. *Infect Immun* **72**: 6806–6816.


