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Toxoplasma gondii transmembrane microneme proteins and their modular design

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Summary

Host cell invasion by the Apicomplexa critically relies on regulated secretion of transmembrane micronemal proteins (TM-MICs). Toxoplasma gondii possesses functionally non-redundant MIC complexes that participate in gliding motility, host cell attachment, moving junction formation, rhoptry secretion and invasion. The TM-MICs are released onto the parasite’s surface as complexes capable of interacting with host cell receptors. Additionally, TgMIC2 simultaneously connects to the actomyosin system via binding to aldolase. During invasion these adhesive complexes are shed from the surface notably via intramembrane cleavage of the TM-MICs by a rhomboid protease. Some TM-MICs act as escorters and assure trafficking of the complexes to the micronemes. We have investigated the properties of TgMIC6, TgMIC8, TgMIC8.2, TgAMA1 and the new micronemal protein TgMIC16 with respect to interaction with aldolase, susceptibility to rhomboid cleavage and presence of trafficking signals. We conclude that several TM-MICs lack targeting information within their C-terminal domains, indicating that trafficking depends on yet unidentified proteins interacting with their ectodomains. Most TM-MICs serve as substrates for a rhomboid protease and some of them are able to bind to aldolase. We also show that the residues responsible for binding to aldolase are essential for TgAMA1 but dispensable for TgMIC6 function during invasion.

Introduction

Toxoplasma gondii is an obligate intracellular parasite of the phylum Apicomplexa, which also includes the deadly agent of malaria, Plasmodium falciparum. Host cell invasion by these parasites is a multi-step process (Carruthers and Boothroyd, 2007) propelled by the gliding motility machinery. It is initiated by apical attachment of the parasite to the host cell, followed by reorientation, formation of a junction between the parasite and host cell membranes, penetration and, finally, sealing of the parasitophorous vacuolar membrane.

Some of the proteins implicated in invasion are sequentially released from two types of secretory organelles, named micronemes and rhoptries (Carruthers and Sibley, 1997). In T. gondii, four complexes composed of soluble and transmembrane microneme proteins or including rhoptry neck proteins (RONs) have been investigated and shown to perform non-overlapping functions during invasion (Fig. 1A). More complexes are, however, likely to contribute to invasion as additional uncharacterized transmembrane microneme proteins (TM-MICs) are encoded in the genome.

The selective participation of each of the four complexes in the invasion process has been uncovered by generating conventional or conditional knockouts of the genes encoding components of the complexes. The TM-MIC TgMIC2 forms a multimeric complex with the soluble partner TgM2AP (Rabenau et al., 2001; Jewett and Sibley, 2004). Parasites depleted in TgMIC2 are markedly deficient in host cell attachment, motility and hence unable to invade host cells (Huynh and Carruthers, 2006). Another complex, composed of the transmembrane protein TgMIC6, is interacting with two soluble molecules TgMIC1 and TgMIC4. Genetic disruption of any of the three encoding genes is still compatible with parasite survival (Reiss et al., 2001) even if the complex has been demonstrated to play an important role in invasion in vitro and to contribute to virulence in vivo (Cerede et al., 2005; Blumenschein et al., 2007; Sawmynaden et al., 2008). A third complex is composed of the TM-MIC TgMIC8 and the soluble protein TgMIC3. Genetic disruption of TgMIC8...
interferes with rhoptry secretion and, consequently, prevents formation of the moving junction (MJ) and completion of invasion (Kessler et al., 2008). A fourth complex, which uniquely localizes to the MJ (Alexander et al., 2005), contains the rhoptry proteins TgRON2, TgRON4, TgRON5 and TgRON8 (Alexander et al., 2005; Straub et al., 2008; Besteiro et al., 2009) and the TM-MIC TgAMA1, which anchors the complex to the parasite plasma membrane (PM). Parasites lacking TgAMA1 efficiently attach to host cells but are defective in rhoptry secretion, fail to create a MJ and are consequently unable to invade host cells (Mital et al., 2005). Gliding motility is not significantly altered in the absence of TgAMA1 or TgMIC8 (Mital et al., 2005; Kessler et al., 2008).

So far, TgMIC2 and TgMIC6 are the only TM-MICS shown to play a crucial role as force-transducers during motility and invasion. TgMIC2 binds to receptor(s) on the host cell surface and establishes simultaneously a connection, via its C-terminal cytoplasmic domain (CTD), with the parasite's actomyosin system, hence powering parasite motility. The CTDs of TgMIC2, TgMIC6 and other members of the thrombospondin-related anonymous

Fig. 1. A. Schematic representation of the four major microneme complexes in T. gondii, as well as of TgMIC12 and TgMIC16, when in the micronemes (top) and on the parasite's surface (bottom). Represented is the currently known composition of the complexes, including the various proteolytic cleavage events as demonstrated in Carruthers et al. (2000) and Harper et al. (2006) for TgMIC2/M2AP, in Meissner et al. (2002), Opitz et al. (2002) and Sawmynaden et al. (2008) for TgMIC6/MIC1/MIC4, in Meissner et al. (2002) and Cerede et al. (2005) for TgMIC8/MIC3, in Hehl et al. (2000), Alexander et al. (2005), Straub et al. (2008) and Besteiro et al. (2009) for TgAMA1/RON2/RON4/RON5/RON8, in Opitz et al. (2002) for TgMIC12 and in this study for TgMIC16.

B. Amino acid sequence alignment of the TMDs and CTDs of TgMIC2, TgMIC6, TgMIC12, TgAMA1, TgMIC16, TgMIC8 and TgMIC8.2. Boxed in grey are the TMDs, in green the putative rhomboid cleavage sites and in pink the motifs for traffic to the micronemes. The tryptophan signature residue boxed in yellow and the acidic residues boxed in blue are both involved in binding to aldolase.
protein (TRAP) family in *Plasmodium* (TRAP, CTRP and TLP) interact with aldolase, a glycolytic enzyme also capable of binding to filamentous-actin (F-actin) (Buscaglia *et al.*, 2003; Jewett and Sibley, 2003; Heiss *et al.*, 2008; Zheng *et al.*, 2009). It is unknown whether other *T. gondii* TM-MICs, that are part of adhesive complexes and exhibiting crucial functions in invasion, can as well interact with aldolase and thus act as bridge molecules.

At the end of the penetration process, the tight interactions formed between the different MIC complexes and the host cell receptors have to be disengaged to let the parasite freely replicate. This has been proposed to occur by proteolytic shedding of the MIC complexes from the parasite’s surface. Cell-based cleavage assays and studies on parasites have demonstrated that critical cleavage events takes place at a conserved motif within the luminal part of the transmembrane domains of TgMIC2, TgMIC6, TgMIC12 and TgAMA1. The protease responsible for this intramembrane cleavage was named microneme protein protease 1 (MPP1) and likely corresponds to a PM intramembrane cleavage (Opitz *et al.*, 2002; Brossier *et al.*, 2003; Urban and Freeman, 2003; Zhou *et al.*, 2004; Howell *et al.*, 2005). Prime candidates for this shedding activity are TgROM4 and TgROM5, which are found at the PM of the parasite (Brossier *et al.*, 2005; Dowse *et al.*, 2005). More recently, parasites depleted in TgROM4 indicate that this protease acts as sheddase for TgMIC2 and TgAMA1 and hence critically contributes to the creation of an apical-posterior gradient of adhesins necessary for an apical orientation of the parasite during invasion (Buguliskis *et al.*, 2010). At the end of the penetration process, the tight interactions formed between the different MIC complexes and the host cell receptors have to be disengaged to let the parasite freely replicate. This has been proposed to occur by proteolytic shedding of the MIC complexes from the parasite’s surface by the TgROM5 activity.

A prerequisite for successful invasion is the correct trafficking of the MIC complexes, from the endoplasmic reticulum, where they are pre-assembled, to the micronemes, where they are stored prior to invasion. Similar to other eukaryotic sorting mechanisms, some TM-MICs are accurately targeted to the micronemes via recognition of a tyrosine-based motif in the cytoplasmic CTDs (Sheiner and Soldati-Favre, 2008). TgMIC2 and TgMIC6 CTDs contain such a microneme targeting motif (EIEYE) and have been shown to serve as escorts for the soluble MICs that are part of the respective complexes (Di Cristina *et al.*, 2000; Reiss *et al.*, 2001; Opitz *et al.*, 2002). Recent studies have also revealed an important contribution of some of the soluble MICs to trafficking. TgMIC1 was shown to promote folding of TgMIC6 by serving as a quality control mechanism (Saouros *et al.*, 2005) and other soluble MICs contain pro-peptides that act as luminal forward targeting elements and are indispensable for correct trafficking of the entire complex (Harper *et al.*, 2006; Brydges *et al.*, 2008; El Hajj *et al.*, 2008).

To gain insight into the mechanistic contribution of each of the MIC complexes to invasion, we have undertaken a detailed analysis of the TM-MICs currently identified in *T. gondii* and included a new member, TgMIC16. We have searched for the presence of trafficking determinants, assessed their susceptibility to intramembrane cleavage and their ability to interact with aldolase. The results indicate that in contrast to TgMIC2, TgMIC6 and TgMIC12, the CTDs of TgMIC8, TgMIC8.2, TgAMA1 and TgMIC16 do not carry the information for proper trafficking to the micronemes and cannot therefore be considered as escorters. All these TM-MICs, apart from TgMIC8.2, appear to be susceptible to intramembrane cleavage and the CTDs of TgMIC6, TgMIC12 and TgAMA1 can bind to aldolase in pull-down assays. Additionally, we have identified specific residues within the CTD of TgAMA1 that are required for both association with aldolase and host cell invasion. Collectively these data support a model describing the involvement of TM-MICs, as part of complexes with distinct and non-overlapping functions during invasion.

### Results

**TgMIC16 is a conserved Coccidia TM-MIC containing six thrombospondin repeat (TSR) domains**

A search in the *T. gondii* genome database for putative new microneme proteins containing TRAP family-like transmembrane sequences led to the identification of a gene encoding a hypothetical protein (TGME49_089630) of 669 amino acids. This gene model (80.m00085) has also been identified by a recent *in silico* screen for secretory proteins and was proposed to reside in an apical compartment (Chen *et al.*, 2008). The amino acid sequence of the protein includes an N-terminal predicted signal peptide, six putative TSR type 1 domains (Fig. S1) and one TMD (transmembrane domain). This TMD is located close to the C-terminal end, contains a motif reminiscent of a rhomboid cleavage site and delimits a very short C-terminal tail (Fig. 2). Another TMD is also predicted at the N-terminal end of the protein (TMHMM prediction programme), but with a low probability and therefore it is not depicted as such in the schemes. A search of the available apicomplexan genomes revealed that homologues of TGME49_089630 are present in the genomes of *Neospora caninum* and *Eimeria tenella* but are absent in Hemoparasida, suggesting that this gene is restricted to the Coccidia. Alignment of the amino acid sequences of these genes (Fig. 2A) uncovered a very similar domain structure.

Transient expression of this new protein carrying a Ty epitope at the C-terminus revealed a micronemal localization in *T. gondii* tachyzoites (Fig. 2B). Reflective of this
localization and the nomenclature status, this protein has been named TgMIC16 (accession number EU791458). Given its predicted domain structure and the presence of a putative rhomboid cleavage site in the TMD, this protein was included in this study along with the TM-MICs that are part of the four major *T. gondii* MIC complexes (TgAMA1, TgMIC2, TgMIC6 and TgMIC8). We also included one of the homologues of TgMIC8, TgMIC8.2, previously known as MIC8-like 1 (Kessler et al., 2008). A chimera of the TgMIC8 ectodomain fused to the TM-CTD portion of TgMIC8.2 was able to functionally complement mic8 ko parasites, indicating that the TM-CTDs of these proteins are functionally equivalent (Kessler et al., 2008). Finally, TgMIC12, the homologue of the *Eimeria* TM-MICs EtMIC4 and EmTFP250 (Witcombe et al., 2004; Periz et al., 2009), shown before to be susceptible to rhomboid cleavage (Opitz et al., 2002), was also included in the comparative analysis.

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Multiple motifs and functions are conserved in the TM-CTDs of the TM-MICs

Several lines of evidence indicate that the TM-CTDs of the TM-MICs play an essential role in supporting the functionality of their respective complexes (information regarding the composition and susceptibility to proteolytic cleavage of these complexes is recapitulated in Fig. 1A), and therefore an alignment of the amino acid sequences of these domains was performed and carefully examined (Fig. 1B).

Previous studies on the aldolase binding capacity of the CTDs of TgMIC2 and other TRAP-related TM-MICs have demonstrated the importance of both a stretch of acidic residues and a penultimate tryptophan residue in the extreme C-terminal sequence (Buscaglia et al., 2003; Starnes et al., 2006). TgMIC6 and TgMIC12 possess both the acidic stretch and a tryptophan residue near the C-terminus (Fig. 1B). The CTDs of the two TgMIC8 homologues possess a penultimate tryptophan residue but are not of acidic nature. Conversely, TgAMA1 contains the C-terminal acidic residues, but the most C-terminal tryptophan is 21 residues in from the C-terminus (W520). This residue lies within a FW motif that is highly conserved in the AMA1 homologues of different apicomplexans (Donahue et al., 2000; Hehl et al., 2000) and is known to be essential for invasion in Plasmodium falciparum (Treckel et al., 2009). The short TgMIC16 CTD does not exhibit any feature of the aldolase-binding motifs.

TgAMA1, TgMIC2, TgMIC6 and TgMIC12 possess a rhomboid cleavage site, IAGG or IAGL, at a conserved position within the TMD, and were previously shown to be cleaved in the parasite and in vitro cleavage assays (Opitz et al., 2002; Urban and Freeman, 2003; Brossier et al., 2005; Dowse et al., 2005; Howell et al., 2005; Buguliskis et al., 2010). A very similar motif is found at the corresponding position within the TMD of TgMIC16 and in a different position within the TMD of TgMIC8. No rhomboid cleavage motif could be identified in the TMD of TgMIC8.2.

From the two motifs shown to be essential for TgMIC2 targeting to the micronemes (Di Cristina et al., 2000), the sequence SYHYY is not conserved in any of the CTDs of the TM-MICs analysed, whereas the motif EIEYE is strictly conserved in the tail of TgMIC6. A sequence resembling EIEYE is similarly positioned in the TgMIC12 and TgAMA1 CTDs but the critical last glutamine residue is only present in TgMIC12. No sequence reminiscent of such a targeting motif could be identified in the CTDs of the TgMIC8 family members or TgMIC16.

Several TM-MICs lack trafficking signals in their CTDs

The TM-CTDs of TgMIC2, TgMIC6 and TgMIC12 were shown to be able to target to the micronemes the surface antigen 1 protein (TgSAG1), lacking its GPI anchor signal (Di Cristina et al., 2000; Reiss et al., 2001; Opitz et al., 2002). From these studies it was concluded that these three TM-MICs act as escorters, bringing the soluble components of their respective complexes to the organelle. TgMIC8 was initially suspected to act as an escorter based on the ability of a GPI anchored TgMIC8 construct to bring TgMIC3 to the PM (Meissner et al., 2002). However this experiment only demonstrated that TgMIC3 and TgMIC8 were part of the same complex and the escorter hypothesis had to be revisited in the light of a recent study, which established that the soluble partner TgMIC3 was correctly targeted to the micronemes, even in the absence of TgMIC8 (Kessler et al., 2008).

To assess if TgMIC8, TgMIC8.2 and TgMIC16 contain trafficking information, their TM-CTDs were C-terminally fused to the SAG1 coding sequence, lacking its GPI anchoring signal, and to a Ty-1 tag epitope, and expressed under the control of the TgMIC2 promoter (the constructs are depicted in Fig. S2). Depending on the information carried by the CTDs, these chimeras were expected to travel through the secretory pathway and to be secreted onto the parasite surface, either via the micronemes by extracellular parasites at the time of invasion, or via the dense granules (DGs) in a constitutive fashion.

In parasites expressing pMS1tyMIC16TM-CTD or pMS1tyMIC8TM-CTD, the fusion proteins accumulated in the DGs and were delivered to the parasitophorous vacuole (PV), as shown by colocalization with the DG marker GRA3 (Fig. 3). An identical SAG1 fusion with the TM-CTD of TgMIC8.2 (pMS1tyMIC8.2TM-CTD) accumulated in the trans-Golgi, as it accumulated in a compartment in the proximity of cis-Golgi as shown by staining with the marker GRASP-YFP (Pelletier et al., 2002) (Fig. 3). Because of the presence of a TMD, the chimeric proteins were expected to accumulate at the PM. The absence of PM staining suggests that these proteins are cleaved once delivered to the PM, and what is being detected is the processed form.

Insight into the trafficking of TgAMA1 was performed by expressing its TM-CTD C-terminally fused to the SAG1 and Ty-1 tag epitope, under the control of the endogenous promoter. The fusion pAS1tyAMA1TM-CTD localized to the DGs, and not to the PM, as previously shown for pMS1tyMIC16TM-CTD and pMS1tyMIC8TM-CTD, suggesting that this protein also undergoes proteolysis (Fig. 4). A series of truncated variants of TgAMA1 with an N-terminal His tag, under the control of the endogenous promoter, were also expressed. Unlike pAS1tyAMA1TM-CTD, pAhisAMA1ΔTM-CTD, encoding the ectodomain only, or pAhisAMA1ΔCTD, encoding the ectodomain and TMD, were predominantly targeted to the micronemes, as...
shown by colocalization with TgMIC4. The same localization was obtained for the full-length protein, pAhisAMA1 (Fig. 4). These data suggest that the ectodomain, but not the CTD, of TgAMA1 assures correct trafficking to the micronemes potentially via interaction with a yet unidentified protein. This is in accordance with the observations made on the *Plasmodium* AMA1 and other micronemal proteins (Healer *et al*., 2002; Treeck *et al*., 2006; 2009).

These results indicate that unlike TgMIC2, TgMIC6 and TgMIC12, none of the other TM-MICs analysed here carry the necessary signal in their CTD to travel to the micronemes.

**Fig. 3.** Localization of several stably transfected chimeras in the parasite using double immunofluorescence analysis and confocal microscopy. Anti-GRASP or GRASP-YFP were used as dense granules and Golgi markers respectively. Scale bars indicate 5 μm. pMS1tyMIC16TM-CTD and pMS1tyMIC8TM-CTD expressing parasites were stained with anti-Ty-1 (in green) and anti-GRASP (in red). pMS1tyMIC8.2TM-CTD was stained with anti-Ty-1 (in red) and colocalized with expression of GRASP-YFP (in green). The nucleus was stained with DAPI (in blue).

**Fig. 4.** Localization of several stably transfected chimeras in the parasite using double immunofluorescence analysis and confocal microscopy and schemes of the different constructs. Anti-MIC4 was used as micronemal marker. Scale bars indicate 5μm. pAS1tyAMA1TM-CTD expressing parasites were stained with anti-Ty-1 (in green) and colocalized with anti-GRASP (in red). pAhisAMA1ΔTM-CTD, pAhisAMA1ΔCTD and pAhisAMA1 expressing parasites were stained with anti-his (in green) and colocalized with anti-MIC4 (in red).
Several TM-MICs are susceptible to cleavage within the membrane-spanning domain

To determine if the SAG1-ty-TM-CTD chimeras were serving as substrates for intramembrane cleavage we generated parasites expressing SAG1 fusion constructs mutated in the predicted rhomboid cleavage sites (the mutated residues are boxed in the schemes depicted in Fig. 5B–E). Analysis by indirect immunofluorescence assay (IFA) of the corresponding transgenic parasite lines revealed that there was a dramatic change in the subcellular localization when compared with the wild-type chimeras (Fig. 3). The mutant fusion proteins accumulated at the PM and residually at the DGs (Fig. 5A), suggesting that they were indeed subject to intramembrane proteolysis and introduction of the mutations conferred resistance to cleavage and accumulation at the parasite surface.

To confirm that the changes in localization coincided with abrogation of cleavage, Western blot analyses were performed on total lysates from transgenic parasites expressing wild-type or mutated SAG1-ty-TM-CTD chimeras (an additional blot can be seen on Fig. S2). pMS1tyMIC16TM-CTD is detectable as a processed form that is no longer detected in the mutant chimera, pMS1tyMIC16mTM-CTD, when the putative rhomboid cleavage site AGGI was mutated to VVLV. The size difference between the processed and non-processed forms suggests that this cleavage is occurring downstream of the Ty-1 epitope, within the putative cleavage motif, pMS1tyMIC16mTM-CTD, when the putative rhomboid cleavage site AGGI was mutated to VVLV. The size difference between the processed and non-processed forms suggests that this cleavage is occurring downstream of the Ty-1 epitope within the TMD (Fig. 5B). Similarly, when the motif IAGG in pMS1tyMIC8TM-CTD was mutated to IILV in pMS1tyMIC8mTM-CTD, there was a change in the migration pattern, compatible with the occurrence of a proteolytic cleavage downstream of the Ty-1 epitope, within the putative cleavage motif (Fig. 5C). In sharp contrast to all the other rhomboid cleavage sites identified to date in apicomplexan sub-cellular localization when compared with the wild-type chimeras (Fig. 3). The mutant fusion proteins accumulated at the PM and residually at the DGs (Fig. 5A), suggesting that they were indeed subject to intramembrane proteolysis and introduction of the mutations conferred resistance to cleavage and accumulation at the parasite surface.

Expression of pMS1tyMIC8.2TM-CTD led to the generation of two products suggesting that the protein undergoes proteolytic maturation (Fig. 5D). The smaller product shows the same migration behaviour on SDS-PAGE as the intramembrane cleavage product observed for pMS1tyMIC16TM-CTD and other fusions (Fig. S2), suggesting that the cleavage occurs within or close to the TMD but there is no recognizable rhomboid cleavage site in the TMD of TgMIC8.2 and therefore we could not test for rhomboid cleavage.

pAS1tyAMA1TM-CTD was mainly detected in the DGs and was subject to proteolysis at a site compatible with intramembrane cleavage as determined by Western blot (Figs 4 and 5E). In fact shedding of TgAMA1 from the parasite surface, during invasion, was previously reported to occur by proteolytic cleavage at a precise site within the TMD (Howell et al., 2005; Bugulis et al., 2010). These results indicate that pAS1tyAMA1TM-CTD, pMS1tyMIC8TM-CTD and pMS1tyMIC16TM-CTD are cleaved likely by a rhomboid protease at the PM.

Several TM-MICs bind to aldolase

To determine whether other TM-MICs besides TgMIC2 can interact with aldolase, we examined the ability of bacterially expressed GST–CTD fusions to bind to aldolase by in vitro pull-down assays. Purified recombinant rabbit aldolase was used as source of aldolase and GST-MIC2CTD and GST alone served as positive and negative controls respectively. The sequences of the TM-MICs used to generate the GST-fusions are listed in Fig. S3. The experiment was repeated several times using independent purifications of each GST-fusion and reproducibly showed that GST-MIC8.2CTD and GST-MIC16CTD were unable to bind to aldolase. In contrast, significant binding was monitored with GST-MIC6CTD, GST-MIC12CTD and GST-AMA1CTD (Fig. 6A), confirming previous results with TgMIC6 (Zheng et al., 2009). In the case of GST-MIC8CTD, no conclusions could be taken regarding binding, because of aberrant migration of the protein on the gel, possibly result of protein instability.

It is known that mutation of the conserved tryptophan residue at the C-terminus of PbTRAP, PITRAP, PTLP and TgMIC2 abrogates interaction with aldolase (Buscaglia et al., 2003; Jewett and Sibley, 2003; Heiss et al., 2008). TgMIC6 possesses a tryptophan residue in the same position as the one in TgMIC2 (Fig. 1B), suggesting that this residue is responsible for binding to aldolase. Indeed a GST-MIC6mCTD, in which W518 was replaced by an alanine residue (MIC6W/A) (Fig. S3), showed a significant reduction in binding to aldolase (Fig. 6B). Although there is not a tryptophan residue at the extreme C-terminus of TgAMA1, site-directed mutagenesis was performed to mutate F519W520, which is more distal to the C-terminus but represents a highly conserved motif in all apicomplexan AMA1 proteins and precedes a stretch of acidic residues in the TgAMA1 CTD (Fig. S3). Intriguingly, the replacement of F519W520 by AA (AMA1FW/AA) led to a significant reduction in the binding of GST-AMA1mCTD to aldolase (Fig. 6A).

Mutations in the CTD of TgAMA1 that block binding to aldolase inhibit invasion

The availability of mutant parasite strains in which the TgMIC6 and TgAMA1 genes have been disrupted by double homologous recombination offered the opportunity to examine the importance of the tryptophan residue in TgMIC6 and TgAMA1 for invasion (Reiss et al., 2001; Mital et al., 2005).
A mutant of TgMIC6, TgMIC6W/A-Ty, was generated in which the residue W348, lying in a similar position as the tryptophan residue involved in TgMIC2 binding to aldolase, was converted to an alanine residue (Fig. 1B). TgMIC6-Ty and TgMIC6W/A-Ty expressing vectors were used to complement the mic6ko strain and the resulting proteins were shown to localize to the micronemes (Fig. 7A). Given that TgMIC6 is acting as escorter, in the absence of the protein, the soluble partners of its adhesive complex, TgMIC1 and TgMIC4, are mistargeted to the DGs and hence unable to participate in the invasion process (Reiss et al., 2001). In consequence, the mic6ko mutant is virtually comparable to a triple-knockout of TgMIC6, TgMIC1 and TgMIC4 (Reiss et al., 2001), in a situation parallel to the mic1ko strain, where TgMIC4 and TgMIC6 fail to traffic to the micronemes. Consistent with the invasiveness of mic1ko (Cerede et al., 2005), mic6ko shows about a 50% reduction of invasion efficiency compared with the RH-2YFP strain, which was used as an internal standard for parasite fitness (Fig. 7B). Complementation of mic6ko with either MIC6Ty or MIC6W/A-Ty restored the invasion phenotype to a level comparable to wild-type level. Gliding assays showed that mic6ko parasites are not defective in gliding and, as expected, the MIC6W/A-Ty complemented parasites also glide normally (Fig. 7C). These results suggest that the residue W348, and therefore aldolase binding, is not critical for the function of the TgMIC4–MIC1–MIC6 complex during invasion.

To study whether the residues F519W520 contribute to the function of the TgAMA1 during invasion we used a previously reported TgAMA1 conditional knockout parasite (ama1ko; Mital et al., 2005), in which the expression of wild-type (myc-tagged) TgAMA1 can be controlled by the addition of anhydrotetracycline (ATc). In the absence of ATc, AMA1myc is expressed in these parasites and they are fully invasive; in the presence of ATc, AMA1myc expression is repressed and the parasites are severely defective in invasion (Mital et al., 2005). The ama1ko parasites were transfected with plasmids encoding Flag-tagged wild-type or mutant TgAMA1 (AMA1WTFlag and AMA1FW/AAFlag, respectively).
tively), and independent clones expressing similar levels of AMA1WTFlag and AMA1FW/AAFlag in the presence of ATc were isolated. Both the wild-type and mutant proteins localized to the apical end of the parasite, as shown by colocalization with M2AP, indicating proper localization (Fig. 8A and data not shown). While AMA1WTFlag was able to complement the ATc-induced invasion defect in the ama1koi parasites, AMA1FW/AAFlag was not (Fig. 8B). These data demonstrate that the hydrophobic residues F519W520 within the CTD of TgAMA1 are essential for both aldolase binding and host cell invasion.
Discussion

MIC complexes serve essential roles during host cell invasion, by mediating parasite attachment, MJ formation and bridging of the host cell receptors to the actomyosin system, hence promoting gliding and invasion. The smooth transition through the various steps of the invasion process requires a high level of coordination not only between the different MIC complexes but also between each component of a given complex. The TM-MICs, in particular are multitaskers and execute distinct functions that are specified by their modular design. The ectodomains, on one hand, recruit microneme or rhoptry proteins to the complex and, in several instances also interact directly with host cell receptors; and the TM-CTDs, on the other hand, contribute to targeting, proteolytic shedding and connection to the actomyosin system of the parasite. TgMIC2 and other members of the TRAP family are suited to carry out these multiple tasks (Morahan et al., 2009).

In this study, we have investigated and compared with TgMIC2, the biological properties of the TM-MICs associated with the three other major MIC complexes known to be involved in invasion, as well as of TgMIC12,
TgMIC8.2 and TgMIC16, whose functions remain to be established.

Targeting to the micronemes, as demonstrated for the rhoptries (Ngo et al., 2003; Richard et al., 2009), resembles post-TGN targeting in other eukaryotes (Sheiner and Soldati-Favre, 2008). Complexes of soluble and TM-MICs are formed in the endoplasmic reticulum and travel through the secretory pathway until they are finally secreted (Reiss et al., 2001; Huynh et al., 2003). Some TM-MICs have been shown to act as escorters, implying that their CTDs are recognized by components of the vesicular sorting machinery (Meissner et al., 2002). Consistent with this idea, two micronemal targeting motifs, SYHYYY and EIEYE, were identified in TgMIC2 (Di Cristina et al., 2000). The apparent absence of such motifs in the CTDs of TgAMA1, TgMIC16 and the TgMIC8 family members is in agreement with the findings here that the respective SAG1-ty-TM-CTD chimeras fail to traffic to the micronemes. Consequently, these TM-MICs do not function as escorters and are likely to interact, via their ectodomains, with other proteins that carry a determinant for micronemal targeting. Consistent with this hypothesis, the chimera the chimera MIC8 fused to the CTD of Plasmodium berghei TRAP localizes to the micronemes (Kessler et al., 2008) although the PbTRAP TM-CTD does not confer trafficking to micronemes in T. gondii (Di Cristina et al., 2000). Similarly, the refined analysis of TgAMA1 clearly established that it is the ectodomain of the protein that carries the necessary traffic information to the micronemes. Studies on AMA1 in P. falciparum led to the same conclusion (Healer et al., 2002; Treeck et al., 2006; 2009). These observations imply that TgAMA1, TgMIC8, TgMIC8.2 and TgMIC16 may belong to complexes that are composed of more than one type of TM-MIC.

The SAG1-ty-TM-CTDs chimeras localized either to the DGs and PV (TgMIC8, TgMIC16 and TgAMA1), or were retained in the Golgi (TgMIC8.2). An alignment of the TM-CTDs of the selected TM-MICs predicted the presence of a rhomboid cleavage motif similar to IAGG in the TMDs of TgMIC2, TgMIC6, TgMIC12, TgAMA1 and TgMIC16. An IAGG motif is also present within the TMD of TgMIC8, but it is significantly shifted within the TMD, closer to the CTD. No apparent rhomboid cleavage site signature could be identified in the TMD of TgMIC8.2. Consistent with cleavage at the rhomboid cleavage motif, proteolytic processing at the expected position was observed for pMS1tyMIC8 TM-CTD, pMS1tyMIC16 TM-CTD and pAS1tyAMA1 TM-CTD chimeric proteins. To provide further evidence for intramembrane processing by a rhomboid, point mutations were introduced in the identified cleavage motifs. The majority of the mutations introduced abrogated processing and thus provided strong evidence that the chimeras are cleaved within their TMD by a rhomboid-like protease. Interestingly, while TgMIC8 appears to be cleaved by a rhomboid, this proteolysis occurs much closer to the cytoplasmic region of the TMD than in all the other TM-MICs. This may allow the direct release of the CTD into the cytoplasm, where it can initiate a signalling cascade, as previously proposed (Kessler et al., 2008).

Given the absence of a recognizable rhomboid cleavage motif in the TMD of TgMIC8.2, we could not assess the nature of the processing event. However, the cleavage product runs at a size compatible with intramembrane processing and TgMIC8.2-CTD can replace that of TgMIC8 (Kessler et al., 2008), which is susceptible to intramembrane cleavage. It is consequently still plausible that the TgMIC8.2 chimera is as well processed within the TMD.

Preventing the proteolytic cleavage by mutagenesis had an anticipated impact on the localization of the SAG1-ty-TM-CTD chimeras. All the mutant chimeras showed a dramatic change in subcellular localization, accumulating at the parasite’s surface, indicative of cleavage abrogation. In a prior study, a similar accumulation at the parasite PM was observed for the uncleaved SAG1TgMIC12 TM-CTD mutant, which was mistargeted to the DGs (Optiz et al., 2002). This observation suggested that MPP1 is a constitutively active rhomboid-like protease at the PM of the parasite. We cannot discriminate between cleavage of the SAG1-ty-TM-CTD constructs at the PM or inside the parasite, but it is likely that TgMIC16 and TgMIC8 fusion constructs are cleaved by MPP1, because the corresponding non-cleaved mutants accumulate at the parasite’s surface. The unambiguous assignment of each of the TM-MIC substrates to a given protease awaits further investigations.

Several Plasmodium TM-MIC proteins have been reported to interact with the F-actin binding protein aldolase and in this way bridge the host cell surface with the actomyosin motor of the parasite. These proteins share the structural features characteristic of TRAP, namely an N-terminal secretion signal, a van Willebrand A-domain, one or more TSR domains, a TMD with a rhomboid cleavage motif and an acidic CTD with a unique tryptophan residue close to the C-terminus (Morahan et al., 2009). In T. gondii, TgMIC6 and TgMIC2 are the only TM-MICs shown to bind to aldolase (Jewett and Sibley, 2003; Starres et al., 2006; Zheng et al., 2009), in a model compatible with TgMIC2 redistribution along the parasite’s surface upon invasion (Carruthers and Sibley, 1999) and demonstrated role in motility and invasion (Huynh and Carruthers, 2006). The patches of acidic amino acids constituting the aldolase binding site within the TgMIC2 CTD (Starres et al., 2006) are not strictly conserved in the TgMIC6, TgMIC12 and TgAMA1 CTDs (Fig. S4) but as shown in this study, these proteins are able to bind to aldolase in an in vitro pull-down assay. This suggests that
the composition in acidic amino acids and their precise location within the CTD can accommodate a level of variation. The second prominent feature of aldolase binding is the presence of a conserved tryptophan residue at the extreme C-terminus of the CTD. All the TM-MICs studied here possess this residue except TgAMA1 and TgMIC16, and as shown by mutation of the residue in TgMIC6, the residue mediates binding to aldolase. Although the TgMIC8 family members possess a tryptophan residue at the extreme C-terminus, the acidic patch is absent and none of these CTDs bind to aldolase in the in vitro assay. This is in accord with functional analysis showing no motility defect in TgMIC8-depleted parasites (Kessler et al., 2008). In contrast, TgAMA1 is able to bind to aldolase without an extreme C-terminal tryptophan, although TgAMA1 does contain a tryptophan just N-terminal to a patch of acidic residues (W520; Fig. S4) within an FW motif that is well conserved among AMA1 homologues in other Apicomplexans (Hehl et al., 2000). Mutation of this FW motif in TgAMA1 disrupted both aldolase binding and invasion. This result suggests that TgAMA1 serves as a bridging protein that physically connects the glideosome (via its CTD) to other components of the MJ complex (via its ectodomain) and thus plays a critical role in the posterior translocation of the MJ complex during invasion. However, during invasion the majority of the TgAMA1 is not restricted to the MJ but is found over the parasite’s surface (Alexander et al., 2005; Howell et al., 2005). This suggests that there may be two pools of TgAMA1 at the parasite surface, one of which is bound to aldolase and is responsible for anchoring the MJ complex to the actomyosin motor. Whether the remaining fraction of TgAMA1 serves a distinct function or is simply available to be recruited to the MJ is unknown, but it is possible that the two pools of TgAMA1 are distinguished by different post-translational modifications of their CTDs, such as phosphorylation (Trecek et al., 2009). Disappointingly, we repeated efforts to monitor TgAMA1, or even TgMIC2, interaction with aldolase in the parasite by co-immunoprecipitation were unfruitful.

The extreme C-terminus of TgMIC12 shares a nearly strictly conserved amino acid sequence with TgMIC2, and this reflects its comparable propensity to bind to aldolase in pull-down experiments. Given the fact that no functional data are available to date on TgMIC12, the physiological relevance of these observations is not known.

TgMIC6 binds less efficiently to aldolase compared with the CTDs of TgMIC2 or TgMIC12, and this can simply reflect the fact that fewer acidic residues are present at its extreme C-terminus. TgMIC6 wild type or TgMIC6 carrying a W548A mutation are both able to functionally complement the invasion defect in parasites depleted of TgMIC6, suggesting that the tryptophan residue may not be crucial for the function of the TgMIC1–MIC4–MIC6 complex.

Consistent with these findings mic6ko showed no defect in gliding motility, suggesting that the function of TgMIC1–MIC4–MIC6 complex in invasion might be assisted via the formation of a macrocomplex by another TM-MIC that connects to the actomyosin system.

It remains to be determined for TgMIC12, if aldolase plays a role in its functions in vivo and if it reflects a direct interaction with the actomyosin motor or another biological role.

Taken together, there is an excellent correlation between the predictions made from sequence analysis and the three biological properties examined experimentally: presence of trafficking determinants, susceptibility to rhomboid protease cleavage and binding to aldolase. Moreover, the assessed properties of the TM-MICs are in good accord with their functional contribution to the individual steps of the invasion process (Table 1).

**Experimental procedures**

**Reagents and parasite culture**

Restriction enzymes were purchased from New England Biolabs and secondary antibodies for Western blots and IFA from Molecular Probes. *T. gondii* tachyzoites (RH strain wild-type and RHhxgypr) were grown in human foreskin fibroblasts (HFF) or Vero cells in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine and 25 μg ml⁻¹ gentamicin. ama1ko parasites were cultured in DMEM supplemented with 1% fetal bovine serum, 25 μg ml⁻¹ mycophenolic acid, 50 μg ml⁻¹ xanthine and 6.8 μg ml⁻¹ chloramphenicol (Mital et al., 2005). Down-regulation of AMA1-myc expression in intracellular ama1ko parasites was achieved by incubation of infected cells for 36 h in medium containing 1,5 μg ml⁻¹ anhydrotetracycline (ATc, Clontech).

**Cloning of DNA constructs**

For determination of MIC16 localization in *T. gondii*, the full-length gene was amplified from tachyzoite cDNA by PCR using the primers 1969 and 1971 (Table S1). The PCR product was purified, digested with EcoRI and NsiI and cloned into the corresponding sites in pTUB8Ty (Meissner et al., 2002).

For expression in *T. gondii* as N-terminal SAG1-Ty fusions, DNA fragments coding for the TMD and CTD of MIC8, MIC8.2 and MIC16 were amplified from tachyzoite cDNA by PCR using the primers 1887 and 1888, 1889 and 1890, 1970 and 1972 (Table S1). MIC8-TrCTD, MIC8, 2TrCTD and MIC16-TrCTD were digested with Sall and Pael, and each fragment was cloned into the corresponding sites in pMSAG1Ty vector, which drives expression...
under control of the TgMIC2 promoter, originating pMSAG1tyMIC8TMCTD, pMSAG1tyMIC8.2TMCTD and pMSAG1tyMIC16TMCTD (Di Cristina et al., 2000).

For expression of the different AMA1 constructs under control of its endogenous promoter in RH strain T. gondii, DNA fragments coding for TMD and CTD, the full-length protein, only the ectodomain (amino acids 1–456) or the ectodomain and TMD (amino acids 1–479) were amplified from tachyzoite cDNA by PCR using the primers 1079 and 1080, 2225 and 1080, 2247 and 2249 and 2247 and 2248 respectively (Table S1). The three last pair of primers added a 8His tag immediately after amino acid 25. AMA1TMCTD was digested with XhoI and PacI and cloned in pMSAG1Ty vector, originating pMSAG1tyAMA1TMCTD, and the other PCR products were digested with Nsil and PacI and cloned into the corresponding sites in pROP1 vector, originating pROPhisAMA1, pROPhisAMA1ΔTM-CTD and pROPhisAMA1ΔCTD (Soldati et al., 1998). The AMA1 promoter was amplified with primers 2462 and 2463 and cloned between KpnI and NsiI sites in the pROP1 vectors, originating pAhisAMA1, pAhisAMA1ΔTM-CTD and pAhisAMA1ΔCTD, or between KpnI and NsiI sites in the pMSAG1Ty vector expressing AMA1 TM and CTD, originating pAS1tyAMA1TM-CTD.

Generation of plasmid pSK+A/AMA1-Flag for expression of Flag-tagged TgAMA1 in the ama1koi parasites has been described elsewhere (F. Parussini, submitted).

For bacterial expression, DNA fragments corresponding to TM-CTDs of TgMIC2, TgMIC12, TgMIC8, TgMIC8.2, TgMIC6, TgMIC6W/A, TgAMA1 and TgMIC16 were amplified from tachyzoite cDNA by PCR using the primers 176 and 177, 1599 and 710, 325 and 326, 1832 and 1833, 211 and 212, 211 and 3100, 1948 and 1949 and 2001 and 2002 respectively (Table S1). PCR products were purified using the Easy Pure-DNA Purification Kit (Biozym). MIC2TMCTD, MIC6TMCTD and MIC6W/A TMCTD were digested with EcoRI and SalI, MIC12TMCTD and AMA1TMCTD with EcoRI and XhoI, MIC8TMCTD with BamHI and MIC8.2TMCTD and MIC16TMCTD with BamHI and XhoI. Each fragment was cloned into the corresponding sites in the pGEX4T1 vector to generate N-terminal GST-fusions.

**Mutated constructs**

To mutate the motif FW to AA on the AMA1CTD of the plasmid pGEX4T1-AMA1TMCTD the primers 1830 and 1831 were used in a site-directed mutagenesis reaction using the commercial QuikChange II Site-Directed Mutagenesis Kit (Stratagen) according to the manufacturer’s instructions. Similarly, the residues AGG, YTG, AG or AGGI were mutated to ILV, VL or VVLV in the TMDs of MIC8, MIC8.2 and MIC16 in the plasmids pMSAG1tyMIC8TMCTD, pMSAG1tyMIC8.2TMCTD and pMSAG1tyMIC16TMCTD respectively, using the primers 2003 and 2004, 2005 and
2006 or 2226 and 2227 respectively. To mutate F519W620 to AA on the AMA1 cytosolic tail in pSK+A/AMA1-Flag, primers TgAMA1FW/AA.f and TgAMA1FW/AA.r (Table S1) were used for site-directed mutagenesis as described above, generating the vector pSK+A/AMA1FW/AAFlag.

**Protein expression and purification**

pGEX-4T1 vectors encoding the MIC2, MIC12, MIC6, MIC8, MIC8.2, MIC16 or AMA1 TMCTD GST-fusion proteins were transformed into the *Escherichia coli* BL21 strain (Novagen, Madison, WI). Protein expression was induced using 1 mM isopropyl-beta-d-thiogalactopyranoside (IPTG) for 4 h at 37°C. Bacterial pellets were resuspended in 1× PBS supplemented with 1 mg ml\(^{-1}\) of Lysozym, 10 μg ml\(^{-1}\) DNAse, 20 μg ml\(^{-1}\) RNase and 1 mM PMSF, and were allowed to homogenize for 30 min at 4°C, following which, cells were disrupted by five consecutive cycles freeze/thaw. After centrifugation (30 min, 30 000 r.p.m.), the supernatant containing the soluble GST-fusions was collected, and purified using GSH-beads (Glutathione Sepharose 4 Fast Flow, Amersham) according to the manufacturers advice.

**GST-fusion protein pull-down experiment**

Glutathione-sepharose beads were incubated with 0.5 mg of GST-fusion proteins or GST for 1 h at 4°C. Beads were washed twice with PBS and once with buffer XB (50 mM Tris-HCl, pH 7.5; 1% (vol/vol) Triton X-100; 0.5% (vol/vol) sodium deoxycholate; 0.2% (wt/vol) SDS; 100 mM NaCl; 5 mM EDTA) containing protease inhibitors (Sigma P8340), added directly to SDS-PAGE-loading buffer and boiled for 10 min. For all other samples, extracts from 2 × 10⁸ parasites were prepared in 1× PBS by five consecutive freeze/thaw cycles with intermediate homogenization, following two consecutive sonications, and the suspension was boiled in SDS-PAGE-loading buffer containing 100 mM DTT. SDS-PAGE was performed using standard methods. Separated proteins were transferred to a nitrocellulose membrane using a semidyry electrobolter. Western blots were performed using anti-Ty1 mAb (Bastin *et al*., 1996), anti-AMA1 mAb (B3.90, Donahue *et al*., 2000), anti-myc mAb (Mital *et al*., 2005) and anti-Flag mAb (Sigma F3165) in 5% non-fat milk powder in 1× PBS. As secondary antibody, a peroxidase-conjugated goat anti-mouse or anti-rabbit antibody was used (Molecular Probes, Paisley, UK). Bound antibodies were visualized using either the ECL system (Amersham Corp) or with SuperSignal West Pico chemiluminescent substrates (Pierce).

**IFA and confocal microscopy**

All manipulations were carried out at room temperature. Intracellular parasites grown in HFF seeded on glass coverslips were fixed with 4% paraformaldehyde (PFA) for 10 min or 4% PFA/0.002% glutaraldehyde for 10 min. Following fixation, slides were quenched in 1× PBS-0.1 M glycine. Cells were then permeabilized in 1× PBS/0.2% Triton X-100 (PBS/Triton) for 20 min and blocked in the same buffer supplemented with 2% FCS (PBS/Triton/FCS). Slides were incubated for 60 min with the primary antibodies anti-GAP45, anti-MIC4 (Brecht *et al*., 2001), anti-Myc, anti-GRA3 (kindly provided by JF Dubremetz) or anti-Ty1 diluted in PBS/Triton/FCS, washed and incubated for 40 min with Alexa488- or Alexa594-conjugated goat anti-mouse or goat anti-rabbit IgGs diluted in PBS/Triton/FCS. DAPI staining was performed with a concen-
treatment of 0.1 mg of DAPI per millilitre of 1× PBS for 5 min incubation before slides were mounted in Fluormount G (Southern Biotech) and stored at 4°C in the dark. Micrographs were obtained on a Zeiss Axioskop 2 equipped with an Axiovision software. Images were recorded and treated on computer through the AxioVision software. Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NTDM/IRB) using a 63 Plan-Apo objective with NA 1.40. Optical sections were recorded at 250 nm per vertical step and four times averaging.

Cell invasion assays

Comparison of different *T. gondii* strains for invasion efficiency was done using an RH-2YFP strain as internal standard. A confluent 60 mm dish of HFF was heavily infected with a mixture of the strain of interest and RH-2YFP parasites. Some hours later the dish was washed to remove any non-invaded parasites. Two days later parasites egressed from their host cells and were collected by centrifugation at 240 g, room temperature for 10 min and resuspended in 5 ml of culture medium (DMEM supplemented with 2 mM L-glutamine, 5% FCS and 25 μg ml⁻¹ gentamicin) preheated to 37°C. From this suspension a 1:10 dilution was made in preheated medium, the ratio of non-YFP to YFP parasites was determined in a Neubauer chamber (ratio between 0.8 and 7), and 500 μl were inoculated into a well on a 24-well IFA plate. Invasion was allowed to take place for 1 h at 37°C; then the wells were washed two times in CM-PBS (1 mM CaCl₂, 0.5 mM MgCl₂ in PBS) and refilled with fresh medium. The plate was incubated for another 24–32 h in order for the parasites to devote. Afterwards cells were fixed with 4% paraformaldehyde for 20 min, followed by 3 min incubation with 0.1 M glycine in PBS to quench the reaction and subjected to an IFA. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked in 2% bovine serum albumin, 0.2% Triton X-100 in PBS for 20 min. The cells were then stained with rabbit anti-TgMLC antibodies followed by Alexa594 goat anti-rabbit antibodies (Molecular Probes). Total number of parasite vacuoles and RH-2YFP parasite vacuoles were counted on 20 microscopic fields on each IFA-slide with a minimum of 750 vacuoles in total per slide. Only vacuoles containing at least two parasites were counted. The ratio of YFP to non-YFP vacuoles was calculated and compared with the ratio obtained from live parasites at the beginning of the experiment. Each experiment has been repeated six times. Alternatively, host cell invasion was measured using a laser scanning cytometer-based assay (Mital *et al*., 2006). Briefly, parasites grown for 36 h in the presence of ATc were harvested, added to HFF monolayers and incubated at 37°C. One hour post infection, the coverslips were fixed, blocked and labelled with an anti-SAG1 antibody (mAb GII-9; Argene, North Massapequa, USA) followed by an R-phycoerythrin-conjugated secondary antibody (‘orange,’ DAKO, Carpenteria, USA). Samples were then permeabilized, blocked and labelled with anti-SAG1 followed by an Alexa647-conjugated secondary antibody (‘red,’ Molecular Probes). Samples were analysed on a CompuCyte Laser Scanning Cytometer equipped with a BX50 upright fluorescence microscope (Olympus America, Melville, USA), 20× objective (N.A. 0.5), argon ion (488 nm) and helium/neon (633 nm) lasers and three filter blocks/photomultiplier tubes [530–555 nm (green), 600–640 nm (orange), and 650 nm (long-red)]. Data were acquired and analysed using Wincyte 3.4 Software (CompuCyte, Cambridge, USA). Red parasites were counted to determine the total number of parasites per field. The number of orange, extracellular parasites was counted and subtracted from the total to calculate the number of invaded parasites per field. One-way ANOVA and Dunnett’s multiple comparison post test were used to determine the significance of differences between groups. *P*-values of less than 0.05 were considered significant.

Gliding motility assay

Freshly egressed tachyzoites were filtered, pelleted and resuspended in calcium-saline containing 1 μM of ionomycin. The suspension was deposited on coverslips previously coated with poly-L-lysine (3 h at room temperature). Parasites were fixed with PAF/GA, and IFA using the TgMLC antibody (mAb GII-9; Argene, North Massapequa, USA) followed by an R-phycoerythrin-conjugated secondary antibody (‘red,’ Molecular Probes). Samples were analysed on a CompuCyte Laser Scanning Cytometer equipped with a BX50 upright fluorescence microscope (Olympus America, Melville, USA), 20× objective (N.A. 0.5), argon ion (488 nm) and helium/neon (633 nm) lasers and three filter blocks/photomultiplier tubes [530–555 nm (green), 600–640 nm (orange), and 650 nm (long-red)]. Data were acquired and analysed using Wincyte 3.4 Software (CompuCyte, Cambridge, USA). Red parasites were counted to determine the total number of parasites per field. The number of orange, extracellular parasites was counted and subtracted from the total to calculate the number of invaded parasites per field. One-way ANOVA and Dunnett’s multiple comparison post test were used to determine the significance of differences between groups. *P*-values of less than 0.05 were considered significant.

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References


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