Microneme protein 8 – a new essential invasion factor in Toxoplasma gondii

Henning Kessler1, Angelika Herm-Götz1, Stephan Hegge1, Manuel Rauch1, Dominique Soldati-Favre2, Friedrich Frischknecht1 and Markus Meissner1,*

1Hygieneinstitute, Department of Parasitology, University Hospital Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany
2Department of Microbiology and Molecular Medicine, University of Geneva, CMU, 1, rue Michel-Servet 1211, Geneva 4, Switzerland

*Author for correspondence (e-mail: markus.meissner@med.uni-heidelberg.de)

Summary
Apicomplexan parasites rely on sequential secretion of specialised secretory organelles for the invasion of the host cell. First, micronemes release their content upon contact with the host cell. Second, rhoptries are discharged, leading to the formation of a tight interaction (moving junction) with the host cell, through which the parasite invades. The functional characterisation of several micronemal proteins in Toxoplasma gondii suggests the occurrence of a stepwise process. Here, we show that the micronemal protein MIC8 of T. gondii is essential for the parasite to invade the host cell. When MIC8 is not present, a block in invasion is caused by the incapability of the parasite to form a moving junction with the host cell. We furthermore demonstrate that the cytosolic domain is crucial for the function of MIC8 and can not be functionally complemented by any other micronemal protein characterised so far, suggesting that MIC8 represents a novel, functionally distinct invasion factor in this apicomplexan parasite.

Introduction
The phylum Apicomplexa consists of several pathogens of immense medical and veterinary importance, such as those causing malaria, toxoplasmosis or coccidiosis. Because they are obligate intracellular parasites, a key step during their life cycle is the invasion of the host cell. Apicomplexan parasites actively invade the host cell in a complex, organised, stepwise process (Carruthers and Boothroyd, 2006) that involves specialised secretory organelles (the micronemes, rhoptries and dense granules) and a unique form of movement termed gliding motility (Soldati and Meissner, 2004). Upon initial attachment to a surface, the parasite starts to glide in a substrate-dependent manner. Gliding involves the discharge of adhesive transmembrane proteins at the apical end by specialised secretory organelles called micronemes. It has previously been demonstrated that micronemal proteins of the TRAP family (thrombospondin-related anonymous protein) are essential for gliding motility and invasion (Huynh and Carruthers, 2006; Sultan et al., 1997) by binding to host-cell receptors and bridging them to the cytoplasmic actin-myosin motor of the parasite via the glycolytic enzyme aldolase (Buscaglia et al., 2003; Jewett and Sibley, 2003; Starnes et al., 2006). The next step in invasion is the recognition of the host cell followed by rhoptry secretion and intimate attachment to the host cell. A conditional-mutagenesis approach for the micronemal protein AMA1 has demonstrated a distinct role for a micronemal protein during invasion. In the absence of AMA1, the parasite fails to form a moving junction (MJ) (Mital et al., 2005). The formation of the MJ depends on the secretion of rhoptry neck proteins (RONs) and their interaction with AMA1 (Alexander et al., 2005; Lebrun et al., 2005). In the absence of AMA1, the RONs are still secreted and associate with the host cell in loose patches but do not form an MJ (Alexander et al., 2005; Boothroyd and Dubremetz, 2008). Up until now, the events leading to rhoptry secretion and subsequently to the formation of the MJ are not well understood. The functional linkage between micronemes and rhoptries led us speculate that additional micronemal proteins might be responsible for the establishment of the MJ.

Micronemal protein 8 (MIC8) was previously identified by sequence similarity to other micronemal proteins in the genome of Toxoplasma gondii. Analysis of MIC8 has demonstrated that it is, like other micronemal transmembrane proteins, secreted upon an increase in intracellular calcium concentration and processed close to its transmembrane domain (TMD). Furthermore, it has been demonstrated that MIC8 interacts with the soluble micronemal protein MIC3 during the transit through the secretory pathway (Meissner et al., 2002a). Here, employing a conditional gene-expression system, we present a detailed functional characterisation of this essential protein (Meissner et al., 2002b). We show that invasion depends on the function of MIC8. Further detailed characterisation of the resulting phenotype showed that this protein does not contribute to gliding motility. Instead it appears that MIC8 plays an essential role in a step before the formation of an MJ. We show, in a novel complementation strategy, that the cytosolic domains (CTDs) of MIC2, PfTRAP or AMA1 cannot substitute the function of the MIC8 CTD, whereas the CTD of the homologous protein MIC8-like1 restores function. This indicates that this transmembrane protein is involved in a novel invasion step leading to formation of the MJ.

Results
Establishment of inducible conditional MIC8 knockout
We employed a tetracycline-inducible system to generate a conditional knockout mutant for mic8. Parasites expressing the tetracycline-dependent transactivator TATi1 (Meissner et al., 2002b) were transfected with a construct encoding a recombinant Ty-tagged
version of MIC8 (MIC8Ty) under control of the Tet-inducible promoters pT7S1 and pT7S4 (Meissner et al., 2001). We generated three independent clones expressing mic8Ty at different levels when grown in the absence of anhydrotetracycline (ATc) (Fig. 1A). The strongest-expressing clone (T7S4-5) showed a partial localisation of MIC8 in an apically localised compartment that corresponded to early endosomes (Fig. 1B, Fig. 2D). By contrast, parasite strains expressing the transgene at a lower level showed a normal localisation of MIC8Ty in the micronemes. Interestingly, prolonged culture in the absence of ATc resulted in loss of transcript expression, indicating that overexpression of MIC8 is unfavourable for the parasite (data not shown).

We removed endogenous mic8 in transgenic parasites (T7S1-4 and T7S4-1) via homologous recombination with the construct MIC8KOCAT (see Materials and Methods) (Fig. 1C) and obtained two independent clonal parasite populations, as shown in analytical PCRs on genomic DNA (Fig. 1D). Both clones, MIC8KOi-T7S1-4 and MIC8KOi-T7S4-1, showed the same behaviour in phenotypic assays (data not shown). We chose MIC8KOi-T7S1-4 for detailed phenotypic analysis and refer to this parasite strain as MIC8KOi.

We analysed the expression of mic8Ty in MIC8KOi. Parasites grown in the absence of ATc showed almost identical levels of expression of MIC8 (~75%, as judged from immunoblots) compared to wild-type parasites (Fig. 2A,B). Incubation of parasites for 48 hours in the presence of ATc led to almost complete ablation of MIC8Ty (to ~2%) compared with parasites grown in absence of ATc for the same amount of time, as judged from immunoblots (Fig. 2A,B and see later). Unless otherwise indicated, parasites were grown in the absence or presence of ATc for 48 hours before the respective experiment was performed. We confirmed that the observed downregulation in mic8 expression was specific, because the expression levels of other micronemal proteins (MIC2 and MIC3) or of alpha-tubulin were not affected (Fig. 2B).

MIC8 is not required for trafficking of MIC3 to the micronemes. Previously, we found that MIC8 and MIC3 are capable of interacting with each other during the transit through the secretory pathway, and we suggested that MIC8 could act as an escorter for MIC3 (Meissner et al., 2002a), in analogy to other identified micronemal escorter proteins, such as MIC6 (Reiss et al., 2001) and MIC2 (Rabenau et al., 2001). To test whether MIC8 is required for correct localisation of MIC3, we analysed localisation of MIC3 in parasites depleted for MIC8. Interestingly, we did not observe a mislocalisation or an altered expression level of MIC3 (Fig. 2B,C). Furthermore, we did not detect any accumulation of MIC3 in early endosomes when parasites overexpressing MIC8 were analysed (Fig. 2D).

Based on these results, we conclude that the interaction between MIC8 and MIC3 is not essential for targeting of MIC3 to the micronemes. To confirm that the observed accumulation of MIC8 in T7S4-5 corresponds to the endosomes, we performed colocalisation analysis with the Golgi marker GRASP-RFP (Pfluger

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**Fig. 1.** Establishment of a conditional knockout for MIC8. (A) Regulation of mic8Ty expression by ATc in three independent parasite strains: T7S4-1, T7S4-5 and T7S1-4. Parasites were grown with (+) or without (−) ATc for 48 hours, fixed and stained with antibodies against Ty, or against MIC6 as a control. Pictures were taken under identical exposure conditions. (B) Immunofluorescence analysis of parasite strains T7S4-1 and T7S4-5 grown for 48 hours in the absence of ATc, as shown in A. Parasites were stained with the indicated antibodies. Strong overexpression of MIC8Ty results in partial accumulation of MIC8 within the secretory pathway (arrows). Other micronemal proteins, such as MIC6, appear not to be affected by overexpression of MIC8. (C) Homologous recombination of endogenous mic8 with a knockout construct in which a CAT-expression cassette is flanked by 1.6 kb (5′FS) and 2.5 kb (3′FS) results in a knockout locus that can be identified using analytical PCR with specific oligonucleotides, as indicated by the arrows. (D) Analytical PCR analysis to verify homologous recombination of the knockout construct in the recipient strains T7S1-4 and T7S4-1. Numbers on top of each lane indicate the respective oligonucleotide combination (see C) used in each PCR reaction. Scale bars: 25 μm (A); 5 μm (B).
Fig. 2. MIC8 is not required for trafficking of MIC3 to the micronemes. (A) Immunofluorescence analysis of MIC8KOi grown for 48 hours in the presence (+) or absence (−) of ATc prior to fixation. Staining was performed with alpha-MIC8, or with alpha-MIC2 as a control (same exposure time for all micrographs). (B) Immunoblot analysis of MIC8KOi and RH parasites grown in the presence or absence of ATc for 48 hours and probed with the indicated antibodies. No significant difference in the expression levels of MIC2, TUB1 or MIC3 could be detected in MIC8KOi cells under the two conditions. (C) Localisation of MIC3 in MIC8KOi parasites grown in the presence of ATc for 48 hours. Parasites were stained with the indicated antibodies. Merged images are shown on the right. Blue stain indicates DAPI (nuclei). (D) Co-localisation studies in the parasite strain T7S4-5, which overexpresses MIC8Ty (see also Fig. 1B). Upper panels: co-localisation study of MIC8Ty and GRASP-RFP (Pfluger et al., 2005) in the parasite strain T7S4-5 transiently transfected with GRASP-RFP demonstrates accumulation of MIC8 in a post-Golgi compartment. Middle panels: co-localisation of MIC8Ty and the propeptide of M2AP (Harper et al., 2006) in early endosomes. Lower panels: co-localisation study of MIC8Ty and MIC3 shows that MIC3 is not localised in early endosomes because of overexpression of MIC8Ty. Right: enlargement of boxed parasite(s) in the merged pictures. Scale bars, 10 μm.

et al., 2005) and found that the majority of retained MIC8 localised apical to the Golgi (Fig. 2D). When we performed co-localisation analysis of MIC8 with an antibody against the propeptide of M2AP, which localises to early endosomes (Harper et al., 2006), we detected retained MIC8 within the same compartment (Fig. 2D).

Phenotypic characterisation of MIC8KOi
To analyse the function of MIC8 in detail, we inoculated human foreskin fibroblast (HFF) cells with parasites in the presence and absence of ATc, and followed the propagation of parasites. Whereas MIC8KOi parasites grown in the absence of ATc showed normal growth when compared to wild-type parasites, no growth was seen when the expression of mic8 was ablated (Fig. 3A).

When we examined infected host cells within 4 days, we occasionally detected huge parasitophorous vacuoles containing large numbers of parasites, indicating that parasites were capable of replicating in the absence of MIC8 (see also supplementary material Fig. S1). Occasionally, we observed lysed vacuoles with freshly egressed motile parasites; these parasites appeared to be incapable of invading neighbouring host cells (Fig. 3A). Several days after the inoculation of cells with parasites, we failed to detect any intracellular parasites, indicating that initially infected host cells had been lysed and that released parasites were incapable of re-invading new host cells (Fig. 3B and data not shown).

Intracellular replication and host-cell egress are not affected by the absence of MIC8
To analyse the function of MIC8 during the asexual cycle of T. gondii, we generated MIC8KOi parasites constitutively expressing GFP. We inoculated HFF monolayers with equal numbers of freshly released MIC8KOi parasites that were grown in the absence of ATc; and subsequently compared growth of the parasites and lysis of initially infected host cells in presence or absence of ATc over a 72-hour time-course (Fig. 3B). We did not see any difference in non-induced host-cell egress: the number of initially infected host cells decreased irrespective of whether ATc was added to the medium or not. Whereas, at 24 hours post-infection, approximately 90% of initially infected host cells were intact, these numbers decreased to approximately 55% and 10% after 48 and 72 hours, respectively (Fig. 3B). However, under conditions of MIC8 depletion, no subsequent infection of neighbouring host cells was detected. This indicates that this protein is specifically required during host-cell invasion, whereas egress is not affected in parasites depleted of MIC8.

Host-cell exit can be artificially triggered by the use of the calcium-ionophore A23187 (Black et al., 2000). When we treated intracellular MIC8KOi parasites grown in the presence or absence of ATc with A23187 for 5 minutes, no significant reduction in egress was detected (supplementary material Fig. S1).

Depletion of MIC8 does not affect parasite attachment or microneme secretion
Initial attachment is the first step in the invasion of the host cell, so we performed attachment assays on fixed host cells. MIC8KOi, TAT1i-expressing and wild-type (RH) parasites grown in the presence or absence of ATc were allowed to attach to the host cell for 15 minutes. After removal of non-attached parasites, the number of parasites associated with the host cell was compared between these groups. We found a slight reduction in attachment when parasites expressing TAT1i were compared with wild-type parasites. Efficiency in attachment appeared to be further decreased in the case of MIC8KOi, irrespective of whether MIC8 was expressed (Fig. 3C). The observed reduction in attachment could be due to the extensive genetic modifications of the parental RH parasites, which required three independent stable transfections in order to generate MIC8KOi. However, no difference in initial host-cell attachment was observed in the case...
Fig. 3. MIC8 is required for parasite survival and is essential during the invasion of the host cell. (A) Upper panels: growth of MIC8KOi parasites was compared to RH parasites using plaque assay. Parasites were inoculated on HFF cells in the presence or absence of ATc for 7 days prior to GIEsMA staining. Lower panels: same experiment as above. Parasites were analysed 72 hours post-infection. Note that in the case of MIC8KOi grown in the presence of ATc, freshly egressed parasites did not invade neighbouring host cells. (B) Analysis of non-induced egress by quantification of intact initially infected host cells. HFF cells were inoculated with MIC8KOi-GFP parasites in the presence or absence of ATc and the decrease in initially infected host cells was assessed over time. The depicted quantification is representative of the results derived from three independent experiments. (C) Attachment assay of freshly released parasites grown in the presence or absence of ATc to fixed cells. Equal numbers of parasites were allowed to attach on host cells at 37°C for 15 minutes before non-attached parasites were removed by consecutive washing steps with cold PBS. Data are mean values of three independent experiments ± s.d. (D) Invasion assay of freshly egressed parasites grown in the presence or absence of ATc as in C. After the indicated time, non-invaded parasites were removed by several washing steps. Intracellular parasites were further incubated for 12 hours and the number of parasitophorous vacuoles was compared. ON, over night. Data shown are mean values of three independent experiments ± s.d. (E) Re-expression of MIC8 in extracellular parasites restores invasion capability. Invasion assay was performed similar to as in D. After MIC8KOi grown in the presence or absence of ATc were allowed to attach for 10 minutes, non-attached parasites were removed. Attached parasites were further incubated in the presence or absence of ATc (−/−: parasites constantly kept in the absence of ATc; +/+: parasites constantly kept in the presence of ATc; +/-: parasites kept in the presence of ATc and further incubated in media without ATc). Data represent mean values of three independent experiments ± s.d.

Of MIC8KOi parasites grown in the presence or absence of ATc. Therefore, MIC8 appears to be not involved in this invasion step (Fig. 3C).

To exclude the possibility that secretion of micronemal proteins was affected, we compared the level of secreted MIC2. We found no difference in secretion of MIC2 in parasites grown in the presence or absence of ATc. Both constitutive microneme secretion as well as induced secretion showed no difference to wild type in the absence of MIC8 (supplementary material Fig. S1). Similar results were obtained with other micronemal proteins, such as MIC6 and MIC4 (data not shown).

MIC8 is required for invasion and not during egress

Next, HFF cells were inoculated with MIC8KOi parasites in the presence or absence of ATc. After host-cell lysis, equal numbers of freshly egressed parasites were analysed for their capability to invade host cells. Parasites were allowed to invade for different durations before the removal of non-invaded parasites and were further incubated in the presence or absence of ATc. We found that parasites depleted of MIC8 were completely deficient in invasion. Even a prolonged invasion time of more than 12 hours did not increase invasion events significantly (Fig. 3D). A direct comparison between MIC8KOi parasites grown in the presence or absence of ATc showed that less then 0.1% of the parasites were invasive. To confirm that MIC8 is a general, essential factor for host-cell invasion, we performed analogous assays on different host-cell types (green monkey kidney cells, HeLa cells and macrophages) and obtained identical results (data not shown).

We were interested to know whether this protein is involved in a signalling cascade during egress of the host cell that prepares the parasite for the next round of invasion, because egress and invasion are tightly linked (Hoff and Carruthers, 2002). If this were the case, extracellular parasites expressing only nascent MIC8 would be incapable of invasion, because no modification of MIC8 can occur during egress. In order to differentiate between a role of this protein during egress and invasion, we modified the above experiment. HFF cells were inoculated for 10 minutes with parasites grown in the presence of ATc. Non-attached parasites were then removed. Subsequently, expression of mic8Ty was reactivated in attached extracellular parasites by the removal of ATc for 12 hours before the invasion of host cells was analysed. Reactivation of MIC8 expression in extracellular parasites restored the capability of invasion (up to 20%) when compared with MIC8KOi parasites that were constantly kept in absence of ATc (Fig. 3E). These results indicate that MIC8 is exclusively involved in invasion and is not required or modified during egress. As soon as a critical level of MIC8 was reached in extracellular parasites, invasion competency was restored. The observed reduction in invasion, when compared to parasites constantly grown in the absence of ATc (Fig. 3E), is probably due to reduced viability of parasites kept for a prolonged time outside of the host cell.

MIC8 is dispensable for gliding motility

Gliding motility is a prerequisite for active invasion of the host cell. Several types of movement can be detected in wild-type parasites (Fig. 4A) (Hakansson et al., 1999). Recently, it has been
Role of MIC8 in *T. gondii* invasion

shown that the micronemal protein MIC2 is essential for helical and twirling motility but not for circular gliding (Huynh and Carruthers, 2006). In the absence of MIC2, parasites show a reduced ability to invade the host cell. To assess whether the observed complete block in invasion upon depletion of MIC8 was due to a reduction in gliding motility, we performed quantitative live imaging. As described previously, three forms of gliding motility can be detected in this assay: circular gliding, upright twirling and helical rotation (Fig. 4A) (Hakansson et al., 1999).

We performed time-lapse microscopy on RH parasites and MIC8KOi grown in the presence or absence of ATc. After image acquisition (one frame/3 seconds for 5 minutes), overlaying projections of the acquired frames revealed the respective motility patterns. All three forms of gliding motility were identified in parasites depleted of MIC8 (Fig. 4A). Although RH parasites showed a higher overall gliding motility than MIC8KOi (30 vs 12% overall gliding motility), no significant reduction in gliding motility was observed between MIC8KOi grown in the presence or absence of ATc (Fig. 4B). When the relative occurrences of the three different forms of gliding were compared, no significant difference between MIC8KOi grown in the presence or absence of ATc was observed (Fig. 4C). The general reduction of gliding motility in MIC8KOi parasites could be due to the extensive genetic modifications of the parental RH parasites (similar to the effect observed for attachment). However, because no difference in gliding motility was evident in the case of MIC8KOi parasites grown in the presence or absence of ATc, the total block in invasion is unlikely to be due to reduced gliding motility in the absence of MIC8.

*T. gondii* expresses two highly conserved homologues of MIC8

Two highly conserved homologues, MIC8-like1 (accession number 76.m01679) and MIC8-like2 (accession number 49m.03396) can be found in the available genome database of *T. gondii* (www.toxoDB.org) (Cerede et al., 2005) (supplementary material Fig. S2). We confirmed expression of MIC8-like1 and MIC8-like2 in *T. gondii* tachyzoites on the RNA level by reverse transcriptase (RT)-PCR (supplementary material Fig. S2). The function of a micronemal transmembrane protein appears to be determined by the interaction of the C-terminal cytosolic tail domain (CTD). In the case of members of the TRAP family, it has been demonstrated that the CTD interacts with the glycolytic enzyme aldolase, which links this protein to the actin-myosin motor essential for gliding motility (Jewett and Sibley, 2003; Buscaglia et al., 2003). An alignment of the CTDs of different microneme proteins indicates that most sequences shown to be essential for interaction with aldolase (Starnes et al., 2006) are not present in the CTD of the MIC8 family members (supplementary material Fig. S2). This corresponds well with our phenotypic characterisation of MIC8KOi that suggests a different function of MIC8 during invasion.

Complementation analysis of MIC8KOi

We were interested to determine whether the CTD of different micronemal proteins can be exchanged for the CTD of MIC8. Therefore, we designed a complementation strategy, taking advantage of the tight regulation and clear invasion phenotype of MIC8KOi, that allowed us to use ATc selection to obtain stable transfected parasites.
Transfection of a construct leading to constitutive expression of a myc-tagged version of MIC8 served as a positive control. Stable selection was performed in the presence of ATc to deplete the parasite of the inducible Ty-tagged copy of MIC8. After 5-6 days, the obtained parasite population was analysed for expression of either the myc- or Ty-tagged version of MIC8. We observed three different sub-populations that expressed MIC8 Ty, MIC8myc or both (Fig. 5A, B) in the presence of ATc, indicating that three independent...
integration events of the complementation construct occurred: exchange of the promoter pT7S1 for pMIC8 (because of homologous recombination of the complementation construct pMIC8MIC8myc with construct T7S1MIC8Ty), random integration of the complementation construct, and a combination of random integration and homologous recombination (Fig. 5A). Only random integration, which resulted in exclusive expression of the myc-tagged complementation construct, indicated functional complementation for MIC8Ty (Fig. 5A).

We generated several complementation constructs in which the CTD and TMD of MIC8 were exchanged for the corresponding domains of MIC2 (accession number AAB63303), AMA1 (accession number AAB65410), PfTRAP (accession number AAA29776), MIC8-like1 or the GPI-anchoring signal of SAG1 (Fig. 5B, upper panel). In the case of MIC2, we generated two different complementation constructs: one including sequences upstream of the TMD that have previously been implicated in the correct processing of MIC2 (Brossier et al., 2003) (Mic2a), and one including only the TMD and CTD (Mic2b). Exclusive expression of the respective complementation construct was only achieved in the case of the construct MIC8myc and in the case of the chimera MIC8mycTMCTDMIC8-like1 (Fig. 5B). In all cases, we verified the correct localisation of the respective chimeric protein in the micronemes (Fig. 5B). Therefore, it appears that the correct targeting of the extracellular domains of MIC8 is not sufficient to restore the function of MIC8. Furthermore, we conclude that the CTD of MIC8 is functionally distinct and cannot be complemented by the CTD of microneme proteins belonging to the TRAP family or of AMA1.

Interestingly, when we generated a mutant in which the highly conserved C-terminal tryptophan of MIC8 was substituted for alanine, we were able to achieve complementation, indicating that this residue, although highly conserved in the MIC8 family (see supplementary material Fig. S2), is not absolutely required for the function of MIC8.

MIC8 is essential for formation of the moving junction

When parasites are treated with cytochalasin D (CD), which leads to an arrest in invasion, an MJ is formed and the content of the rhoptries is secreted into the cytosol of the host cell, leading to the formation of evacuoles (Fig. 6A) (Hakansson et al., 2001). To

![Fig. 6. MIC8 is essential for the formation of the moving junction.](image-url)

(A) Formation of evacuoles is abolished in parasites depleted of MIC8. MIC8KOi, grown in the presence or absence of ATc, was incubated with host cells in the presence of CD. Formation of evacuoles (green) and an MJ (red) was analysed with monoclonal antibodies against Rop2-5 and polyclonal antibodies against RON4. No evacuoles were observed when MIC8KOi was grown in the presence of ATc. Blue staining indicates DAPI (nuclei). (B) Extracellular RON4 was detected by immunofluorescence analysis on non-permeabilised parasites expressing cytosolic GFP as a viability marker. MIC8KOi parasites were allowed to attach to host cells in the presence or absence of CD for 20 minutes. Red indicates an MJ. (C) Quantification of extracellular RON4, the presence of which indicates the formation of an MJ. Approximately 200 parasites were analysed in each assay (error bars indicate s.d. obtained from five independent assays). The units on the y-axis are percent of parasites with RON4 signal. (D) Depletion of MIC8 does not affect RON4 expression levels. Immunoblots from MIC8KOi and RH parasites, grown for 48 hours in the presence (+) or absence (–) of ATc, show efficient downregulation of MIC8 expression in the presence of ATc. No differences in RON4 expression levels could be detected. Scale bars: 15 μm (A); 20 μm (B).
determine whether parasites depleted of MIC8 were capable of forming an MJ and of secreting their rhoptries. MIC8KOi parasites were grown in the presence or absence of ATc, treated with CD and inoculated on HFF cells to determine vacuole formation. In the absence of MIC8, no formation of vacuoles was detected (Fig. 6A). A similar phenotype has been reported for a conditional knockout of the micronemal protein AMA1. Parasites depleted of AMA1 still secrete RONs but fail to establish an MJ between the parasite and the host cell (Alexander et al., 2005; Mital et al., 2005). In this case, RONs associated with the MJ cannot be seen, but rather they localise in loose patches on the surface of the host cell. We were interested to determine whether a similar phenotype could be observed upon depletion of MIC8.

To analyse secretion and localisation of RONs, we used RON4 as a marker. MIC8KOi parasites expressing GFP as a viability marker were grown in the presence or absence of ATc. Parasites were treated with or without CD before inoculation of HFF cells. Subsequently, analysis of RON4 secretion was performed in immunofluorescence assays on non-permeabilised parasites (Fig. 6B). In the absence of MIC8, the occurrence of a RON4 signal was reduced by more then 90% (Fig. 6C) irrespective of whether the parasites were treated with CD. Ablation of MIC8 had no influence on the expression level of RON4 (Fig. 6D). Together, these results demonstrate a crucial role of MIC8 during establishment of the MJ that is upstream of AMA1.

Discussion

Host-cell invasion by apicomplexan parasites is a multi-step process, requiring the sequential secretion of specialised secretory organelles and the action of distinct sets of proteins (Carruthers and Boothroyd, 2006). Whereas secretion of micronemes has been demonstrated to depend on a calcium-signalling cascade, the signals leading to secretion of the rhoptries and formation of the MJ have not been identified so far. Given the functional linkage of microneme and rhoptry secretion, we speculated that micronemal proteins might be implicated in this role.

To shed light on the function of MIC8, we generated a conditional knockout for MIC8 by employing a tetracycline-inducible transactivator system (Meissner et al., 2007). For the generation of transgenic recipient strains with a second controllable copy of mic8, it was necessary to keep expression of the transgene turned off to prevent any deleterious effects due to overexpression of mic8Ty. Interestingly, a strain overexpressing MIC8Ty showed a partial accumulation of this protein in a post-Golgi compartment that corresponded with early endosomes, as shown by co-localisation with the pro-peptide of M2AP (Harper et al., 2006). It is likely that, under conditions of MIC8 overexpression, a factor involved in quality control or maturation of this protein is limiting, resulting in partial accumulation of MIC8 in early endosomes. A similar phenomenon was observed upon overexpression of MIC2 (Huynh and Carruthers, 2006).

It has previously been demonstrated that MIC8 interacts with MIC3 during the transit through the secretory pathway (Meissner et al., 2002a) and it was speculated that MIC8 might act as an escort for MIC3, similar to other micronemal transmembrane proteins (Rabenau et al., 2001; Reiss et al., 2001). However, depletion of MIC8 in the established MIC8KOi parasites did not result in any mislocalisation of MIC3, demonstrating that the observed interaction between MIC8 and MIC3 is not essential for the targeting of MIC3 to the micronemes. Currently, we can only speculate on the mechanism involved in this targeting. It is possible that MIC8 and MIC3 are sorted to the micronemes by interacting with the same protein complex, that MIC3 contains sorting signals that ensure its transport to the micronemes independently of MIC8 or that the escorter function of MIC8 is redundant. Further experiments are required to clarify this complex issue.

We examined crucial events during the asexual life cycle of the parasite and found that depletion of MIC8 explicitly affects the ability of the parasite to invade the host cell. We did not observe any effect on replication, egress or initial attachment to the host cell. Interestingly, reactivation of MIC8 expression in extracellular parasites led to restoration of invasion potency. This shows that MIC8 is not required during egress of the host cell and that the invasion of parasites depends on a critical level of MIC8 expression. Furthermore, parasites depleted for this essential invasion factor showed normal attachment and gliding motility, which is in sharp contrast to the function determined for MIC2 (Huynh and Carruthers, 2006).

We noticed that parasites depleted of MIC8 did not form any vacuoles, similar to a conditional mutant for AMA1 (Alexander et al., 2005; Mital et al., 2005). Parasites depleted for AMA1 are unable to form an MJ and it has been demonstrated that interaction of AMA1 with RONs is essential for this invasion step (Boothroyd and Dubremetz, 2008). However, in the absence of AMA1, RONs are still released by the parasite and can be detected at the surface of the host cell (Alexander et al., 2005). In sharp contrast, we were not able to detect any signal for RON4 when intact non-permeabilised parasites were analysed for RON4 secretion. This indicates that MIC8 functions in an essential step during invasion that is downstream of MIC2 but upstream of AMA1 function.

The absence of RON4 on the surface of the host cell suggests that MIC8 is either required for the initial association of RONs with the surface of the host cell or for rhoptry secretion. In the first case, this role could be facilitated by function of the extracellular domains of MIC8. However, we demonstrated in a novel complementation approach that correct targeting of chimeric proteins consisting of the extracellular domains of MIC8 and the TMD and CTD of different micronemal proteins, such as MIC2, AMA1 and TRAP, did not restore the function conferred by wild-type MIC8. This demonstrates that correct targeting of the extracellular domains is not sufficient to restore the function of MIC8.

Having established that the CTDs of MIC8 and MIC8-like1 determine a novel functional role, we can now continue to identify the cytosolic factors interacting with this domain to gain more mechanistic insights into its function during the establishment of the MJ. Given the clear phenotype of MIC8KOi in invasion, it appears that MIC8-like1 and MIC8-like2, which we show here to be expressed at the RNA level, cannot compensate for the loss of mic8 in tachyzoites.

Previously, it has been shown that MIC8 is capable of binding to the host cell upon dimerisation (Cerede et al., 2002). It is therefore tempting to speculate that, upon dimerisation, MIC8 is involved in a signalling cascade that ultimately leads to rhoptry secretion. Similar mechanisms have been described in other eukaryotes for numerous non-tyrosine kinase receptors that trigger receptor-mediated endocytosis and subsequently a signalling cascade (Li, 2005). In fact, we have recently demonstrated that Rab11A, a small G-protein essential for recycling endocytosis and associated with the rhoptries (Bradley et al., 2005), is required during invasion of the host cell (Herm-Gotz et al., 2007), indicating a link between receptor-mediated endocytosis, receptor recycling and rhoptry secretion.
In conclusion, here, we present evidence that the micronemal protein MIC8 of *T. gondii* plays an essential role during a distinct step of host-cell invasion required for the early formation of the MJ. The characterisation of MIC8 as a key determinant for invasion demonstrates that micronemal proteins are involved in several independent steps during invasion that are likely to be conserved in apicomplexan parasites. The identification of each individual step will not only broaden our understanding of fundamental apicomplexan biology but might also lead to the identification of new drug and vaccine candidates. The challenging issue now will be to further elucidate the mechanistic action of MIC8, especially the role of the MIC8 CTD, which we show here to be essential for its function.

Materials and Methods

Parasite strains, transfecution and culture

*T. gondii* tachyzoites (RH bgprt) and TATI-1 (Meissner et al., 2002b) were grown in HFF cells and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal cell serum (FCS), 2 mM glutamine and 25 μg/ml gentamycin. Selection based on chloramphenicol resistance or pyrimethamine resistance was performed as described previously (Kim et al., 1993; Donald and Roos, 1993).

Generation of the inducible MIC8 knockout

To introduce a Ty-tag into mic8 and generate a vector allowing inducible expression of this gene, the cDNA of mic8 was cloned into a vector p7TetO1 and pTetO4 downstream of the inducible promoter (Meissner et al., 2001). Next, inverse PCR using oligonucleotides MIC8Ty-as (antisense) (5′-CTTAATTAAGATGCTAC-TAGGGGTGCGTGCAGTGACCTGCTGGC-3′) and MIC8s-sense (sense) (5′-CCAGATATCAGTCAGCGAAGTGGC-3′). The PCR fragment was subsequently digested with XhoI and re-ligated, resulting in the expression vectors T75MIC8Ty and T75MIC8Ty, respectively. In order to generate stable parasites with each construct, co-transfection of 60 μg T75MIC8Ty or T75MIC8Ty with 30 μg pDHFR (Donald and Roos, 1993) was performed. The knockout construct was based on the vector p230CAT (Soldati and Booyoth, 1995), in which the 5UTR was amplified using oligonucleotides MIC8-1 (5′-GGGGTATACCTGGGCGTCCTGGC-3′) and MIC8-2 (5′-GGGGTAC-TGGGCGTGCAAGAAAG-3′) and the 3′UTR with MIC8-3 (5′-GGGGCAGATC-TAATTAGCATAAGCCTGCTGGC-3′) and MIC8-4 (5′-TCCCCGCGCGATGAAACAATCTGCG-3′) and MIC8-5 (5′-CCCCGGCACGATGGAAACAATCTGCG-3′) and placed within XhoI and BamHI (Not) restriction sites, respectively. 30 μg of the pMIC8KOCAT construct was transfected and, subsequently, CAT selection was performed. MIC8KOi parasites were isolated from the obtained stable pool of transfectants via limiting dilution and identified via genomic PCR using insert-, genome- and integration-specific oligonucleotide primers. MIC8KOi parasites were then verified in immunoblots and immunofluorescence assays to confirm inducible expression of MIC8Ty. For a subset of studies, MIC8KOi was transfected with pSRT7GFP and GFP-expressing parasites were isolated based on green fluorescence.

Generation of complementation constructs

For the generation of complementation constructs encoding the extracellular domains of MIC8 fused to the TMD and CTD of MIC2, AMA1, PFPTRAP, MIC8/2, RON4. Equal numbers of GFP-positive parasites (~200) were analysed for RON4.

Immunofluorescence and immunoblot

For all experiments, images were captured at one frame per 3 seconds using Axiovision AxioVs40, 16 magnification (objective 10×, Optovar 1.6×). Videos were analysed as described previously (Kuhne et al., 2001). Images were imported and combined in Adobe Photoshop and presented in Adobe Illustrator. To identify different stages of gliding movement, the type of gliding/movement was counted by observing every individual parasite. On average, 447 parasites/movie (101-1052 parasites/movie) were monitored and enumerated for all strains. Using ImageJ 1.34r software, projections of individual parasite. On average, 447 parasites/movie (101-1052 parasites/movie) were monitored and enumerated for all strains. Using ImageJ 1.34r software, projections of individual parasite were collected for all strains. The motion was extracted by observing every individual parasite. On average, 447 parasites/movie (101-1052 parasites/movie) were monitored and enumerated for all strains. Using ImageJ 1.34r software, projections of individual parasite. On average, 447 parasites/movie (101-1052 parasites/movie) were monitored and enumerated for all strains. Using ImageJ 1.34r software, projections of individual parasite. On average, 447 parasites/movie (101-1052 parasites/movie) were monitored and enumerated for all strains. Using ImageJ 1.34r software, projections of individual parasite.
PBS for 20 minutes and blocked in 3% bovine serum albumin in PBS for 20 minutes. The cells were then treated with the primary antibodies, as indicated in the text, for 1 hour followed by Alexa-Fluor-594-conjugated anti-rabbit or Alexa-Fluor-488-conjugated goat anti-mouse antibodies (Protein Labs). Images were taken using Zeiss (Axiovert 200M) and processed using Image J 1.34e software.

SDS page was performed as described previously (Laemmli, 1970). Freshly detergent-free lysates were harvested by centrifugation at 300 g for 10 minutes and lysed in RIPA buffer (150 mM NaCl 1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM EDTA). Equal numbers of parasites were applied per lane on a 10% polyacrylamide gel. Transfer to nitrocellulose and immunoblot analysis was performed as described previously (Reiss et al., 2001).

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