Trans-genera reconstitution and complementation of an adhesion complex in Toxoplasma gondii

HUYNH, My-Hang, et al.

Abstract

Eimeria tenella and Toxoplasma gondii are obligate intracellular parasites belonging to the phylum Apicomplexa. In T. gondii, the microneme protein TgMIC2 contains two well-defined adhesive motifs and is thought to be a key participant in the attachment and invasion of host cells. However, several attempts by different laboratories to generate a knockout (KO) of TgMIC2 have failed, implying that TgMIC2 is an essential gene. As Eimeria and Toxoplasma utilize the same mechanisms of invasion and have highly conserved adhesive proteins, we hypothesized that the orthologous molecule in Eimeria, EtMIC1, could functionally substitute in Toxoplasma to allow a knockout of TgMIC2. TgMIC2 is partnered with a protein called TgM2AP, which corresponds to EtMIC2 in Eimeria. Because the activity of TgMIC2 is most likely tightly linked to its association with TgM2AP, it was thought that the activity of EtMIC1 might similarly require its partner EtMIC2. EtMIC1 and EtMIC2 were introduced into T. gondii, and the presence of EtMIC1 allowed the first knockout clone of TgMIC2 to be obtained. The TgMIC2 KO resulted in significantly decreased [...]
Trans-genera reconstitution and complementation of an adhesion complex in *Toxoplasma gondii*

My-Hang Huynh,1 Corinna Opitz,2 Lai-Yu Kwok,3 Fiona M. Tomley,4 Vern B. Carruthers1* and Dominique Soldati2,3

1Johns Hopkins Bloomberg School of Public Health, W. Harry Feinstone Department of Molecular Microbiology and Immunology, Baltimore MD, USA.
2ZMBH, University of Heidelberg, Germany.
3Imperial College London, Department of Biological Sciences, London UK.
4Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire, UK.

Summary

*Eimeria tenella* and *Toxoplasma gondii* are obligate intracellular parasites belonging to the phylum Apicomplexa. In *T. gondii*, the microneme protein TgMIC2 contains two well-defined adhesive motifs and is thought to be a key participant in the attachment and invasion of host cells. However, several attempts by different laboratories to generate a knockout (KO) of TgMIC2 have failed, implying that TgMIC2 is an essential gene. As *Eimeria* and *Toxoplasma* utilize the same mechanisms of invasion and have highly conserved adhesive proteins, we hypothesized that the orthologous molecule in *Eimeria*, EtMIC1, could functionally substitute in *Toxoplasma* to allow a knockout of TgMIC2. TgMIC2 is partnered with a protein called TgM2AP, which corresponds to EtMIC2 in *Eimeria*. Because the activity of TgMIC2 is most likely tightly linked to its association with TgM2AP, it was thought that the activity of EtMIC1 might similarly require its partner EtMIC2. EtMIC1 and EtMIC2 were introduced into *T. gondii*, and the presence of EtMIC1 allowed the first knockout clone of TgMIC2 to be obtained. The TgMIC2 KO resulted in significantly decreased numbers of invaded parasites compared to the parental clone. In the absence of TgMIC2, TgM2AP was incorrectly processed and mistargeted to the parasitophorous vacuole instead of the micronemes. These findings indicate that the EtMIC1 can compensate for the essential requirement of TgMIC2, but it cannot fully functionally substitute for TgMIC2 in the invasion process or for supporting the correct maturation and targeting of TgM2AP.

Introduction

*Toxoplasma gondii* is an obligate intracellular parasite that infects approximately one-third of the world’s population, with higher rates of infection depending upon the country and culinary preferences (Hill and Dubey, 2002). Healthy individuals infected with *T. gondii* are generally asymptomatic, although minor muscle pain, headaches and enlarged lymph nodes may sometimes occur (Dubey, 1994). Infections are most serious in congenitally infected newborns and in immunocompromised patients, such as transplant recipients and those infected by HIV, where it can cause fatal encephalitis or pneumonia (Luft and Remington, 1992; Remington et al., 1995). *Toxoplasma gondii* belongs to the phylum Apicomplexa, which includes other parasites of medical or veterinary importance, such as *Plasmodium* spp., *Eimeria* spp. and *Neospora* spp.

Apicomplexans share many similarities, including a remarkably conserved mechanism of active host cell invasion. During invasion, the parasites sequentially secrete proteins from three organelles located in the apical complex, the micronemes, rhoptries and dense granules (Carruthers and Sibley, 1997). The micronemes secrete their contents upon host cell recognition by the parasite. Sequencing of microneme proteins from several genera shows that many contain conserved adhesive motifs found in vertebrate adhesion proteins, raising the possibility that these proteins facilitate host-cell attachment as well as function in gliding motility (Tomley and Soldati, 2001). Secretion of the rhoptry contents follows microneme secretion, during the act of invasion, and can be detected in the lumen and membrane of the parasitophorous vacuole (PV) that forms around the parasite. Lastly, the dense granules secrete proteins both during and following the formation of the PV, modifying it for intracellular survival and replication.

The contents of the micronemes are released onto the parasite surface and have been suggested to contribute to the broad host cell specificity of this promiscuous pathogen (Carruthers et al., 1999; Carruthers and Sibley, 1999). Many micronemal proteins contain motifs that are
capable of participating in host cell attachment, such as integrin-like A1 domains, thrombospondin-like repeats, epidermal growth factor-like domains, and lectin-like domains (Fourmaux et al., 1996; Wan et al., 1997; Garcia-Réguet et al., 2000; Reiss et al., 2001; Meissner et al., 2002b). Evidence from several studies implicates the importance of micronemal proteins in invasion (Carruthers and Sibley, 1997; Carruthers and Sibley, 1999; Brossier et al., 2003). However, other than for apical membrane antigen 1 (AMA-1), antibodies to microneme proteins examined did not inhibit parasite invasion (Hehl et al., 2000). Similarly, knockouts of several microneme proteins do not show a marked invasion phenotype (Reiss et al., 2001). Among the proteins secreted from the micronemes following the initial contact with host cells is the TgMIC2/TgM2AP complex. In a recent study, we characterized a knockout of TgM2AP in which an approximately 80% decrease in invasion efficiency was observed in the absence of TgM2AP (Huynh et al., 2003). This provided the first genetic evidence that a microneme protein or complex plays a significant role in the invasion process in T. gondii. In these knockout parasites, TgMIC2 expression was reduced and secretion impaired, with a proportion retained in the ER/Golgi.

Although TgMIC2 has long been thought to play an important role during invasion, its exact function remains unclear as attempts by several laboratories to obtain a knockout have failed. These results, together with studies of the Plasmodium berghei orthologue of TgMIC2, PbTRAP, which showed that it is critical for parasite gliding motility and invasion (Sultan et al., 1997; Kappe et al., 1999), provided strong evidence that TgMIC2 is an integral component of the invasion mechanism and is likely an essential gene.

TgMIC2 belongs to the TRAP family of conserved adhesive proteins, whose members are characterized by a variable number of thrombospondin 1 (TSP-1)-like repeats, as well as conserved transmembrane and C-terminal regions. TgMIC2 contains five TSP-1 repeats and a sixth degenerate repeat. Orthologues of TgMIC2 have been described in four other apicomplexan genera, Cryptosporidium, Eimeria, Plasmodium and Neospora (reviewed in Tomley and Soldati, 2001). An alignment of the thrombospondins, F-spondin, properdin, and lectin-like domains (Fourmaux et al., 1996; Wan et al., 1997; Garcia-Réguet et al., 2000; Reiss et al., 2001; Meissner et al., 2002b). Evidence from several studies implicates the importance of micronemal proteins in invasion (Carruthers and Sibley, 1997; Carruthers and Sibley, 1999; Brossier et al., 2003). However, other than for apical membrane antigen 1 (AMA-1), antibodies to microneme proteins examined did not inhibit parasite invasion (Hehl et al., 2000). Similarly, knockouts of several microneme proteins do not show a marked invasion phenotype (Reiss et al., 2001). Among the proteins secreted from the micronemes following the initial contact with host cells is the TgMIC2/TgM2AP complex. In a recent study, we characterized a knockout of TgM2AP in which an approximately 80% decrease in invasion efficiency was observed in the absence of TgM2AP (Huynh et al., 2003). This provided the first genetic evidence that a microneme protein or complex plays a significant role in the invasion process in T. gondii. In these knockout parasites, TgMIC2 expression was reduced and secretion impaired, with a proportion retained in the ER/Golgi.

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Given the conserved molecules, mechanism of invasion, and the high degree of similarity between TgMIC2 and EtMIC1, this study sought to determine whether EtMIC1 could substitute for TgMIC2. The expression of EtMIC1 allowed genetic disruption of TgMIC2, indicating that it could compensate for the essential requirement for TgMIC2. We demonstrate that this knockout affects expression and processing of other micronemal proteins, parasite invasion efficiency, and virulence in experimental mouse infections.

Results

Expression of EtMIC1 allows disruption of TgMIC2

Eimeria tenella MIC1 and T. gondii MIC2 share conserved structural domains and are likely to perform similar tasks in their respective organisms. We therefore hypothesized that EtMIC1 expression in T. gondii could compensate for TgMIC2 and would then allow us to obtain a knockout of TgMIC2. The parental strain used was RH/hprt, to which EtMIC1 was introduced together with a plasmid containing chloramphenicol acetyltransferase (CAT) allowing selection by chloramphenicol to obtain clone RH/EtM1. TgMIC2 was disrupted in RH/EtM1 by gene replacement with hypoxanthine xanthine guanine phosphoribosyl transferase (HPRT) conferring resistance to mycophenolic acid/xanthine to obtain clone EtM1/TgM2KO. To this clone, EtMIC2 was introduced by random integration, using DHFR-TS mutant gene conferring resistance to pyrimethamine as a selectable marker to obtain EtM1/TgM2KO/EtM2. The strains of parasites generated and the exogenous proteins introduced in this study are summarized in Table 1 and Fig. 1A. Previous attempts in introducing EtMIC1 in T. gondii resulted in accumulation of EtMIC1 in the ER/Golgi, possibly due to the absence of proper targeting sequences (M. White, pers. comm.). The transmembrane and cytoplasmic regions of TgMIC2 fused with the ectodomain of EtMIC1 were thus used in the hope of circumventing improper targeting and difficulties in data interpretation. Figure 1B is an alignment of EtMIC1, TgMIC2 and the EtMIC1 hybrid molecule used in this study.

Proper expression and secretion of EtMIC1 and EtMIC2

To verify that clones expressed exogenous copies of EtMIC1 and EtMIC2, Western blots were performed on whole cell lysates prepared from each clone. Steady state expression levels of TgMIC2 were similar in RH and in RH/EtM1 (Fig. 2A, top panel), whereas levels of EtMIC1 were comparable in RH/EtM1, EtM1/TgM2KO, and EtM1/
Toxoplasma adhesion

TgM2KO/EtM2 (second panel). Figure 2A (third panel) confirms that EtMIC2 is expressed only in the EtM1/ TgM2KO/EtM2 strain; approximately equal loading in all lanes was verified using dense granule protein TgGRA1 expression (bottom panel). It is important to note that the EtMIC1 and EtMIC2 Western blots were run under different conditions (non-reducing and reducing, respectively) and are different exposures. Therefore, the relative quantities of EtMIC1 and EtMIC2 cannot be directly compared.

To determine that the proper protein associations were maintained in these clones, co-immunoprecipitation experiments were performed. TgMIC2 was found to be

Table 1. Genotypes and phenotypes of clones used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>RH</td>
<td>Clinical isolate</td>
<td>HPRT</td>
<td>TgMIC2 +/- HPRT</td>
<td>Sabin (1941)</td>
</tr>
<tr>
<td>RH/EtM1</td>
<td>RH</td>
<td>hprt(pEtMIC1)</td>
<td>TgMIC2/-HPRT</td>
<td>Donald et al. (1996)</td>
</tr>
<tr>
<td>EtM1/TgM2KO</td>
<td>RH/EtM1</td>
<td>hprt(TgMIC1+mic2)-hprt/ EtMIC1 blocking</td>
<td>TgMIC2/-HPRT/CAT</td>
<td>This study</td>
</tr>
<tr>
<td>EtM1/TgM2KO/EtM2</td>
<td>EtM1/TgM2KO</td>
<td>EtMIC1-HPRT/ DHFR-TS(pEtMIC2)</td>
<td>TgMIC2/-HPRT/CAT/ EtMIC2/DHFR-TS</td>
<td>This study</td>
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Fig. 1. Manipulation of adhesive protein complexes.

A. Schematic diagram showing the proteins expressed in each strain used in this study. RH strain parasites lacking HPRT were transfected with EtMIC1 and an RH/EtM1 clone was obtained by chloramphenicol drug selection. The knockout plasmid, pmic2koHPRT, contains the HPRT expression cassette for drug selection and flanking sequences of TgMIC2 to mediate double-crossover homologous recombination at the TgMIC2 locus. The EtM1/TgM2KO strain was obtained by transfection with a pT230/CAT vector containing 5’ and 3’ flanking sequences of TgMIC2 gene and selection with chloramphenicol. EtM1/TgM2KO/EtM2 was obtained by co-transfection of pT8EtMIC2Ty with pDHFR-TS resistant to pyrimethamine.

B. Alignment of the amino acid sequences of EtMIC1, TgMIC2 and the hybrid protein EtMIC1-TgMIC2TMCD, containing the ectodomain of EtMIC1 and the transmembrane and cytoplasmic domains of TgMIC2. The red peptide represents the myc-tag and the blue peptide represents amino acids inserted at the junction of the chimera due to cloning.

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associated with TgM2AP in the RH/EtM1, as it is in the RH parental clone (Fig. 2B, top panel). The complex of EtMIC1 and EtMIC2 was also re-established in T. gondii parasites (Fig. 2B, bottom panel).

We next examined whether the exogenous EtMIC1 and EtMIC2 proteins were properly targeted to the micronemes by immunofluorescence assays. In the RH/EtM1 clone, most of the EtMIC1 was localized to the micronemes, as shown by the co-localization with TgMIC2 (Fig. 2C, top row). However, despite the use of the TgMIC2 cytoplasmic region, a small fraction of EtMIC1 was retained in an area anterior to the nucleus, likely the Golgi apparatus. Expression of TgMIC2 was examined in a mixed population before cloning EtM1/TgM2KO. The micrographs show both non-knockout (TgMIC2 positive, red) and knockout (TgMIC2 negative) parasites showing localization of EtMIC2 in the micronemes, with an additional fraction retained in the Golgi (arrows).

D. Western blot analysis of ESA of untreated (–) or BAPTA-AM-treated (+) parasites shows that EtMIC1 is secreted through the micronemes (top panel), while some EtMIC2 is secreted in both (–) and (+) ESA of the EtM1/TgM2KO/EtM2 strain (middle panel). GRA1 expression is used to show equal loading (bottom panel).
Toxoplasma adhesion 775

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localized with another microneme protein, TgMIC6, but a greater proportion of EtMIC2 was retained in the Golgi compared to EtMIC1 (Fig. 2C, bottom row).

To test whether clones were able to secrete EtMIC1 and EtMIC2 via the micronemes followed by proteolytic release from the parasite surface, excreted/secreted antigen (ESA) fractions were isolated. EtMIC1 was secreted in equivalent levels in RH/EtM1 and EtM1/TgM2KO clones, with slightly lower levels in the EtM1/TgM2KO/EtM2 (Fig. 2D, top panel). Microneme secretion is dependent upon an increase in intracellular calcium and is inhibited by the calcium chelator BAPTA-AM (Carruthers et al., 1999), which is used to distinguish between secretion via the micronemes versus alternative pathways. Secretion of EtMIC1 from all three clones was sensitive to BAPTA-AM treatment, indicating secretion via the micronemes. Interestingly, a fraction of EtMIC2 was insensitive to BAPTA-AM treatment in EtM1/TgM2KO/EtM2 parasites, suggesting that a portion of EtMIC2 is not secreted through the micronemes (Fig. 2D, middle panel); TgGRA1 expression demonstrates both insensitivity to BAPTA-AM treatment and approximate equal loading in all lanes (bottom panel).

To further examine the localization of the non-micronemal fraction of EtMIC2, co-immunofluorescence studies with EtMIC1, a dense granule protein (TgGRA3), and TgMIC6 were performed. A large proportion of EtMIC2 co-localized with EtMIC1 in the micronemes, indicating secretion via the micronemes. Interestingly, a fraction of EtMIC2 was insensitive to BAPTA-AM treatment in EtM1/TgM2KO/EtM2 parasites, suggesting that a portion of EtMIC2 is not secreted through the micronemes (Fig. 2D, middle panel); TgGRA1 expression demonstrates both insensitivity to BAPTA-AM treatment and approximate equal loading in all lanes (bottom panel).

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at least some EtMIC2 is likely mistargeted to the dense granules, with a large proportion retained in the ER/Golgi. Co-localization images with another micronemal protein (TgMIC6) highlight the targeting of EtMIC2 to the micronemes as well as the retention in the ER/Golgi. A potential explanation for this is that EtMIC2 could be expressed in excess of its binding partner EtMIC1, leading to a pool of EtMIC2 that does not contain targeting residues for the micronemes. A Western blot of EtMIC1 and EtMIC2 in *T. gondii* and *E. tenella* cell lysates confirmed that there is an imbalance in the expression levels of EtMIC1 and EtMIC2 (Fig. 3B). By direct quantification of chemiluminescent blots, steady state levels of EtMIC2 were approximately 105% of the levels of EtMIC1 in *E. tenella*, whereas in EtM1/TgM2KO/EtM2 levels of EtMIC2 were 258% of EtMIC1. Results represent four independent experiments, and as indicated in the graph in Fig. 3B, show a statistically significant difference in the levels of EtMIC1 and EtMIC2 (*P* = 0.0223). The slower migration of EtMIC2 in the EtM1/TgM2KO/EtM2 compared to *E. tenella* is the result of a Ty-1 tag in the C-terminus of the exogenous EtMIC2. Collectively, these data suggest that in EtM1/TgM2KO/EtM2, EtMIC2 is expressed in molar excess of EtMIC1, resulting in limited retention in the ER/Golgi and partial secretion via dense granules.

**Loss of TgMIC2 alters TgM2AP processing and secretion**

To determine how the introduction of the EtMIC molecules and the loss of TgMIC2 affected other micronemal proteins, Western blot analyses of cell lysates and ESA (with or without BAPTA-AM treatment) were performed. TgM2AP contains a propeptide that is normally processed in a post-ER secretory compartment, resulting in a 40 kDa product (Rabenau *et al.*, 2001). In the RH/EtM1 clone, TgM2AP was processed normally (Fig. 4A). In EtM1/TgM2KO however, a smaller processed form of TgM2AP was observed. Using antibodies directed against either the propeptide or the C-terminal end of TgM2AP, we verified that this product resulted from N-terminal cleavage

![Fig. 4. TgM2AP is mistargeted and misprocessed in the absence of TgMIC2.](image-url)

A. Western blot analysis of tachyzoite cell lysates with a rabbit anti-TgM2AP antibody shows the mature and the propeptide-processed forms in the RH and RH/EtM1 lysates (top panel). The EtM1/TgM2KO is processed to a smaller product, and there is a larger percentage of proTgM2AP compared to RH. TgM2AP processing is completely abolished in EtM1/TgM2KO/EtM2. Processing of other microneme proteins examined (TgMIC5, TgMIC6 and TgMIC10) are unaffected. Open arrowheads indicate unprocessed forms and black arrowheads represent proteolytic products.

B. Western blot analysis of ESA from BAPTA-AM-treated and untreated parasites with a rabbit anti-TgM2AP antibody shows that secretion of the 43 kDa unprocessed form is BAPTA-AM-insensitive in all strains. The C-terminal processed products in the EtM1/TgM2KO are also smaller than the corresponding products in RH and RH/EtM1.

C. Confocal immunofluorescence staining of a mixed population of EtM1/TgM2KO and non-knockout parasites with TgM2AP and TgMIC2, TgMIC6, or TgGRA3 shows punctate staining of TgM2AP in the micronemes, as well as vacuolar accumulation. Co-staining of TgM2AP and EtMIC2 in EtM1/TgM2KO/EtM2 shows that TgM2AP largely accumulates in the PV.

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of the full-length protein, further downstream from the endogenous cleavage site (data not shown). In addition to a smaller cleavage product, a greater proportion of TgM2AP remained unprocessed in the EtM1/TgM2KO clone. The introduction of EtMIC2 completely abolished propeptide processing of TgM2AP, as shown by detection of only the 43 kDa unprocessed form. A possible explanation for this is discussed below. The misprocessing of TgM2AP did not appear to be a global effect as the expression and processing of other micronemal proteins TgMIC5, TgMIC6, and TgMIC10 in cell lysates was unaffected (Fig. 4A).

Western blot analysis of ESA showed that secretion of the pro-form of TgM2AP (unprocessed) is not blocked by BAPTA-AM treatment in all strains, indicating that this form is secreted through a non-micronemal pathway (Fig. 4B, top bands in all lanes). The top bands in each lane were verified to be proTgM2AP by Western blot analysis with antipropeptide TgM2AP (data not shown). The BAPTA-AM-insensitive secretion of proTgM2AP in the EtM1/TgM2KO represents approximately 25% of the TgM2AP found in the cell lysates, whereas this percentage increases to 80% in the EtM1/TgM2KO/EtM2 strain, likely reflecting the loss of propeptide processing and subsequent mistargeting in this strain. The mature form of TgM2AP is C-terminally processed to four additional products upon secretion from the micronemes (Rabenau et al., 2001), as shown in the untreated ESA of both RH and RH/EtM1. Because the initial processed form of TgM2AP is smaller in EtM1/TgM2KO, subsequent processed forms in this strain were proportionately smaller than the corresponding products in the RH parental strain. Despite improper processing, these products remained BAPTA-AM sensitive, indicating that they were probably targeted to and secreted from the micronemes. Indeed, partial co-localization of TgM2AP with TgMIC6 in EtM1/TgM2KO (Fig. 4C, second row) showed that a subfraction of TgM2AP was properly targeted to the micronemes. However, some TgM2AP was also secreted into the PV, as indicated by partial co-localization with TgGRA3. This PV staining is likely caused by secretion of proTgM2AP presumably via the dense granules. A similar pattern was observed in the EtM1/TgM2KO/EtM2, in which TgM2AP is found largely in the PV.

Loss of TgMIC2 significantly decreases invasion efficiency

TgMIC2 is hypothesized to play an integral role in the process of invasion. We therefore assayed the transgenic parasites to determine whether EtMIC1 could compensate for TgMIC2 during invasion. To examine the invasion efficiency of these strains, a red/green assay was used, which allows attached/extracellular parasites to be distinguished from invaded/intracellular ones. Representative images of the red/green assay of each strain are shown in Fig. 5A, whereas Fig. 5B summarizes the results from three independent experiments, with two replicates of each strain within each experiment. Although the RH/EtM1 strain appeared to have a greater number of attached and invaded parasites, the difference was not statistically significant because of variation in the assay. However, a highly significant decrease in the number of invaded parasites in the EtM1/TgM2KO strain was found, compared to both RH and RH/EtM1 (P = 0.0056 and P = 0.0064, respectively). The numbers of attached EtM1/
TgM2KO parasites, though, were similar to the parental clone (RH). The introduction of EtMIC2 did not recover the invasion deficiency of parasites lacking TgMIC2 ($P = 0.0008$ and $P = 0.0034$ compared to RH and RH/EtM1, respectively). However, EtM1/TgM2KO/EtM2 showed a significant increase in the number of attached parasites compared to RH alone ($P = 0.0095$). BAPTA-AM or cytochalasin D (CytD)-treated parasites were used as controls for an attachment (BAPTA-AM) or invasion (CytD) phenotype, as the former inhibits microneme secretion and subsequent host cell attachment while the latter inhibits invasion by disrupting the parasite’s actin-based motility system. These results demonstrate that EtMIC1 and EtMIC2 are capable of only partially substituting for TgMIC2 and TgM2AP in tachyzoite invasion.

**EtM1/TgM2KO is attenuated in virulence**

Type I strains of *T. gondii* such as RH are extremely virulent in mice; inoculation of a single viable tachyzoite can initiate a fatal infection (Pfefferkorn and Pfefferkorn, 1976). To assay the virulence of strains, approximately 10–50 tachyzoites were injected intraperitoneally into BALB/c mice (Fig. 6A). Six mice were injected per strain in each experiment; the data presented are a summary of two experiments. The RH*hprt* strain was lethally virulent, with all injected mice dying by nine days post injection (square line). The RH/EtM1-injected mice had an approximately 1–2 days delay in death, with one mouse surviving until 15 days post injection (circle line). However, the most striking survival curve was observed with mice injected with the EtM1/TgM2KO strain, in which the first mortality occurred 3 days after the first observed in the RH strain. Importantly, 17% of injected mice were still alive 43 days post injection (inverted triangle line). The survival curve of mice injected with EtM1/TgM2KO/EtM2 was very similar to that of mice injected with RH/EtM1, with a slight delay in death and a single mouse surviving until the end of the observation period (asterisk line). Mice were observed up to 6 weeks post injection, with Western blot analysis confirming the seroconversion of surviving mice 3 weeks after infection. The numbers of infective parasites injected per experiment were verified by plaque assays (Fig. 6B). These results show that TgMIC2 is required for full-scale virulence of RH strain tachyzoites.

**Discussion**

Evidence from numerous studies has implicated the importance of microneme proteins in the process of host cell attachment and invasion by *T. gondii* parasites (reviewed in Carruthers, 2002). Micronemal proteins are secreted at the time of host cell recognition and initial attachment (Carruthers and Sibley, 1997; Garcia-Réguet *et al*., 2000; Rabenau *et al*., 2001). Several lines of evidence indicate that one protein complex, TgMIC2/TgM2AP, is potentially vital in successful rapid host cell invasion. The presence of adhesive motifs on TgMIC2 strongly implies that this complex participates in host receptor recognition and binding, an essential step in the invasion process (Wan *et al*., 1997; Carruthers *et al*., 1999). A recent study characterizing a TgM2AP knockout strain supports this hypothesis. In the absence of TgM2AP, expression of TgMIC2 was reduced and secretion was impaired, with a proportion being retained in the ER/Golgi (Huynh *et al*., 2003). The TgM2APKO parasites showed a significant reduction in an assay testing invasion efficiency, with a decrease of approximately 80% compared to control parasites. Until the current study, it provided the strongest genetic evidence that this complex is integral in *T. gondii* attachment and invasion.

Although a knockout of TgMIC2 would provide the most direct evidence for its functions, concerted efforts by multiple laboratories, including attempts in this study (D. Soldati, pers. comm.), have failed to result in *TgMIC2* disruption. However, data from null mutation studies of PbTRAP, a TgMIC2 orthologue in *P. berghei* parasites, alludes to the role of TgMIC2 in *T. gondii*; mutant parasites...
showed severely disrupted motility, invasion and virulence (Sultan et al., 1997; Kappe et al., 1999). The current study exploited the conserved molecules involved in attachment and invasion between T. gondii and E. tenella by utilizing the orthologue of TgMIC2, EtMIC1, to examine TgMIC2 function. The finding that disruption of TgMIC2 was achieved after introducing EtMIC1 suggests that TgMIC2 and EtMIC1 are indeed functional orthologues within the TRAP family of adhesive proteins. Conservation of the TRAP family within the Apicomplexa underscores the central role of TRAP proteins in the biology of these obligate intracellular parasites.

The decrease in EtM1/TgM2KO invasion efficiency observed could in part explain the delay in death and decreased virulence observed in EtM1/TgM2KO-infected mice, as this delay offers the mouse immune system additional time to mount a defence. It is less clear why RH/EtM1 mice survive longer than control RH-injected mice. Growth assays on the strains showed that there were no differences after 17 h and 26 h of growth, therefore ruling out growth rates as a basis for the observed differences in virulence (data not shown). TgMIC2 secretion into the ESA was partially decreased in the RH/EtM1 strain (data not shown), offering a possible explanation for the observed difference in survival. Inadequate secretion of TgMIC2 may decrease the numbers of parasites capable of invading host cells, thereby requiring more time to build a lethal parasite load. It is also important to note that there are large intrinsic differences between in vitro assays such as the red/green invasion assay and in vivo mice studies. The differences in cell types, tissues, receptors, activation of various pathways in the live organism, et cetera make it difficult to directly correlate the observations in the respective systems.

Functional complementation studies have been used extensively in various areas of study, in which orthologues from one model organism are integrated and analysed in another. Examples include the rescue of a lethal phenotype of the yeast gene SEC62 by the Drosophila melanogaster orthologue (Noel and Cartwright, 1994) or the partial rescue of a knockout in a Saccharomyces cerevisiae premRNA splicing protein with the human equivalent (Lindsey and Garcia-Blanco, 1998). These studies are valuable in several ways, for example, they are used to: (i) examine whether the orthologue can rescue a mutant or knockout; (ii) study the conservation of function/activity of one molecule in another species; or (iii) reconstruct a protein family and study the diversification following speciation or duplication events (Lindsey and Garcia-Blanco, 1998; Hansen et al., 2000; Wang et al., 2000; Gibert, 2002). The present study is unique in that the conserved structure of one orthologue was necessary in order to knock out a presumed essential gene, and the exogenous protein was assayed for the ability to functionally comple-
Experimental procedures

Host cells and Toxoplasma culture

Tachyzoites of RH strain T. gondii were maintained by growth on monolayers of human foreskin fibroblasts (HFF) or on African green monkey (Vero) cells, grown in Dulbecco’s Modified Eagles Medium (DMEM; Gibco) containing 5 or 10% fetal calf serum (Gibco). A clonal isolate of the RH parasites were maintained by growth on Vero cells, grown in DMEM/20 mM Hepes/2% ethanol in a 1.5 ml eppendorf tube (4°C). A clonal isolate of the RH parasites were transfected and selected as previously described (Donald et al., 1996), with the following modifications. Parasites were transfected with 80–100 μg of linearized plasmid. Selection for the presence of chloramphenicol acetyltransferase (CAT) was done as previously described (Kim and Boothroyd, 1993). Stable transformants obtained under chloramphenicol selection were cloned by limiting dilution 6 days after the beginning of chloramphenicol treatment. Selection for the presence of HPRT marker gene was achieved by exposure to mycophenolic acid/xanthine (MPA/X) and cloning of the parasites 3–5 days later by limiting dilution in 96-well microtitre plates containing HFF cells in the presence of MPA/X. To generate TgMIC2 knockout recombinants, four transfections using 10^4 freshly released parasites with 25, 50, 75 or 100 μg of linearized plasmid were conducted in parallel. Pools of stable transformants were analysed by IFA for the absence of TgMIC2. Parasites stably expressing EtMIC1 were obtained by co-transfection of pM2EtMIC1 with the vector expressing CAT, pTub5CAT (pT75R230) (Soldati and Boothroyd, 1995). Parasites stably expressing EtMIC2 were obtained by co-transfection of pT8EtMIC2Ty with a vector expressing TgDHFR-TS containing three mutations conferring resistance to pyrithymethane pDHFR-TSM2M3M4 (Donald and Roos, 1993). Co-transfections were done using restriction enzyme mediated insertion (Black et al., 1995) and a 1/10 molar ratio of selectable vector versus expression vector was used.

Cell lysate and ESA preparation

Induced secretion assays were performed by prewarming 100 μl of DMEM/20 mM Heps/2% ethanol in a 1.5 ml eppendorf tube to 37°C and then adding 100 μl of filter-purified tachyzoites (4 × 10^6 ml^-1 in DMEM + 20 mM Heps). Secretion was induced for 2 min at 37°C and was stopped by placing the tube on ice for 5 min. Culture supernatants (ESA) and parasite cell lysates were collected by centrifugation (1000 g, 10 min, 4°C, twice). The cell pellets were resuspended in 200 μl of DMEM/20 mM Heps/5% SDS-PAGE sample buffer while 0.25 volumes of 5x sample buffer were added to ESA fractions. Samples were boiled for 3 min before loading onto SDS-PAGE gels and Western blotting as described previously (Wan et al., 1997).

For quantitative Western blotting, E. tenella and T. gondii cell lysates were run on 10% SDS-PAGE gels and Western blotted with mouse anti-EtMIC1 (11P-2) and rabbit anti-EtMIC2. After
adding Supersignal substrate (Pierce), chemiluminescence on the blots were captured on a Fujiﬁlm LAS-1000 CCD camera system and analysed using the Fujiﬁlm Image Gauge software.

**Co-immunoprecipitation**

Parasites from each clone were isolated from one T25, ﬁltered and chased with DMEM + 20 mM Heps, and pelleted at 1000 r.p.m. at RT. The parasites were resuspended and lysed for 30 min in 1 ml of RIPA buffer (50 mM Tris pH 7.5, 1% Triton X-100, 5% Na deoxycholate, 0.2% SDS, 100 mM NaCl, 5 mM EDTA pH 8.0). Lysates were centrifuged for 10 min at 4°C and 13 000 r.p.m., and 200 μl used per co-immunoprecipitation. Lysates were first precleared with 10 μl of washed protein G-sepharose for 1 h at 4°C, to which either 2 μl mAb EtMIC1 (11P-2) or 50 μl mAb TgMIC2 (8E9) were added and rotated at 4°C overnight. Ten microlitres of washed protein G-sepharose beads were added to each cell lysate and incubated for 1 h at RT. Beads were washed for 5 min in RIPA buffer and pelleted at 3000 r.p.m. for 5 min; this was repeated three times. Beads were resuspended in 1× sample buffer, boiled for 3 min, vortexed, boiled and vortexed again, and centrifuged at 13 000 r.p.m. for 2 min. Samples were run on a 10% SDS-PAGE gel and Western blotting as described above, using either rabbit anti-TgM2AP or rabbit anti-EtMIC2.

**Indirect immunofluorescence microscopy**

All manipulations were carried out at room temperature. Tachyzoite-infected HFF cells on glass coverslips were ﬁxed with 3% paraformaldehyde-0.05% glutaraldehyde or 4% paraformaldehyde only, for 20 min, followed by 3 min incubation with 0.1 M glycine in PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked in 2% FCS or bovine serum albumin in PBS for 20 min. The cells were then stained with the primary antibodies followed by Alexa 594 goat anti-rabbit or Alexa 488-conjugated goat anti-mouse antibodies (Molecular Probes, Cappel and Bio-Rad). Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM/IRB) using a 100× Plan-Apo objective with NA 1.30. Single optical sections were recorded with an optimal pinhole of 1.0 (according to Leica instructions) and 16 times averaging. Other micrographs were obtained with a Zeiss Axioskop equipped with a CCD camera (Photometrics Type CH-250). Adobe Photoshop (Adobe Systems, Mountain View, CA) was used for image processing.

**Red/green invasion assay**

Red/green invasion assays were performed as described previously (Huynh et al., 2003). Briefly, HFF monolayers were infected with 1 × 10⁶ parasites/ chamber for 15 min, ﬁxed, and external (attached) parasites were stained with rabbit IgP30 (SAG1) before Triton X-100 permeabilization and detection of internal (invaded) parasites with a monoclonal antibody against SAG1. Secondary antibodies used were Oregon green goat anti-mouse and Texas red goat anti-rabbit (Molecular Probes, Eugene OR). DAPI (5 μg ml⁻¹ Sigma) was added to the secondary antibody solution to stain host nuclei. Data were compiled from three independent experiments, each from counting six ﬁelds/clone at 600× total magniﬁcation. Fields were randomly selected (operator moved the microscope stage without viewing the sample) in the same pattern for all samples and cell counts were made in a blinded fashion.

**In vivo virulence assay**

Groups of 5–6 BALB/c mice were infected intraperitoneally with 20–50 tACHyoites of RH, EtM1/TgM2KO and EtM1/TgM2KO/ EtM2 mutant parasites. The numbers of parasites injected were estimated by plaque assays performed in parallel. The survival of mice after infection was monitored over a period of 6 weeks, and seroconversion of all surviving mice was conﬁrmed by West- ern blot analysis 3 weeks after infection. Results of two indepen- dent experiments are presented.

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