Functional analysis of rhomboid proteases during Toxoplasma invasion

Bang Shen  
Washington University School of Medicine in St. Louis  
Jeffrey S. Buguliskis  
Washington University School of Medicine in St. Louis  
Tobie D. Lee  
Washington University School of Medicine in St. Louis  
L. David Sibley  
Washington University School of Medicine in St. Louis

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Please let us know how this document benefits you.

Recommended Citation
https://digitalcommons.wustl.edu/open_access_pubs/3402
Functional Analysis of Rhomboid Proteases during *Toxoplasma* Invasion


Updated information and services can be found at:
http://mbio.asm.org/content/5/5/e01795-14.full.html

http://mbio.asm.org/content/5/5/e01795-14.full.html#SUPPLEMENTAL

This article cites 52 articles, 18 of which can be accessed free at:
http://mbio.asm.org/content/5/5/e01795-14.full.html#ref-list-1

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more>>
Functional Analysis of Rhomboid Proteases during Toxoplasma Invasion

Bang Shen, Jeffrey S. Buguliskis, Tobie D. Lee, L. David Sibley
Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA

ABSTRACT Host cell invasion by Toxoplasma gondii and other apicomplexan parasites requires transmembrane adhesins that mediate binding to receptors on the substrate and host cell to facilitate motility and invasion. Rhomboid proteases (ROMs) are thought to cleave adhesins within their transmembrane segments, thus allowing the parasite to disengage from receptors and completely enter the host cell. To examine the specific roles of individual ROMs during invasion, we generated single, double, and triple knockouts for the three ROMs expressed in T. gondii tachyzoites. Analysis of these mutants demonstrated that ROM4 is the primary protease involved in adhesin processing and host cell invasion, whereas ROM1 or ROM5 plays negligible roles in these processes. Deletion of ROM4 blocked the shedding of adhesins such as MIC2 (microneme protein 2), causing them to accumulate on the surface of extracellular parasites. Increased surface adhesins led to nonproductive attachment, altered gliding motility, impaired moving junction formation, and reduced invasion efficiency. Despite the importance of ROM4 for efficient invasion, mutants lacking all three ROMs were viable and MIC2 was still efficiently removed from the surface of invaded mutant parasites, implying the existence of ROM-independent mechanisms for adhesin removal during invasion. Collectively, these results suggest that although ROM processing of adhesins is not absolutely essential, it is important for efficient host cell invasion by T. gondii.

IMPORTANCE Apicomplexan parasites such as Toxoplasma gondii express surface proteins that bind host cell receptors to aid invasion. Many of these adhesions are subject to cleavage by rhomboid proteases (ROMs) within their transmembrane segments during invasion. Previous studies have demonstrated the importance of adhesion cleavage for parasite invasion and proposed that the ROMs responsible for processing would be essential for parasite survival. In T. gondii, ROM5 was thought to be the critical ROM for adhesion shedding due to its robust protease activity in vitro and posterior localization on the parasite surface. Here, we knocked out all three ROMs in T. gondii tachyzoites and found that ROM4, but not ROM5, was key for adhesion cleavage. However, none of the ROMs individually or in combination was essential for cell entry, further emphasizing that essential pathways such as invasion typically rely on redundant pathways to ensure survival.

Toxoplasma gondii is an obligate intracellular pathogen infecting a wide range of animals as well as humans and can cause severe complications in immunocompromised individuals (1). Belonging to the phylum Apicomplexa, T. gondii shares with other members of this large group of parasites a common set of structures and mechanisms for host cell invasion (2). For the apicomplexan parasites that are of clinical and veterinary significance, such as Plasmodium, Toxoplasma, and Cryptosporidium, the invasion process provides a potential target for antiparasitic drug and vaccine design. However, this potential is largely unmet due to limited knowledge on the essential components of this process.

Host cell invasion by apicomplexan parasites is a multistep process that includes gliding motility, host cell attachment, and active penetration (2). Parasites contain specialized secretory organelles called micronemes and rhoptries that release proteins to mediate invasion (3). Micronemes discharge soluble and transmembrane proteins (referred to as MICs) to the parasite surface, where they form adhesive complexes (MIC2/M2AP [MIC2-associated protein], MIC3/MIC8, MIC1/MIC4/MIC6, etc.) that bind host receptors to mediate gliding motility and host cell attachment (4). After initial host cell binding, which is mediated by micronemal proteins, parasites secrete their rhoptry contents into host cells (5). Rhoptry proteins found in the neck of the organelle (referred to as RONs [rhoptry neck proteins]) form the moving junction (MJ) (6, 7), a crucial structure for invasion that is formed by close opposition of host and parasite membranes and through which the parasite enters the host cell.

Micronemal adhesins undergo complex proteolytic processing after exocytosis (8). In T. gondii, three types of proteases, historically termed microneme protein protease 1 (MPP1), MPP2, and MPP3, are involved in processing adhesins (8). MPP2 trims the N terminus of MIC2 (9) and the C termini of M2AP and MIC4 (10). MPP3 cleaves M2AP and differs from MPP2 in terms of sensitivity to protease inhibitors (10). The specific proteases mediating
ROMs also show unusual kinetics that do not depend on substrate accessibility but rather are driven by access of the substrate to the catalytic site (22). Collectively, these features suggest that ROMs have been coopted from an earlier quality control system for detecting nonnative substrates, typically rich in Ala and Gly (18, 19). Because they are not within their transmembrane domains (15–17).

Rhomboids recognize a cluster of helix-breaking residues near the external surface of the transmembrane domain of their substrates, typically rich in Ala and Gly (18, 19). Because they are not highly sequence specific, ROMs often work on substrates across widely different taxa, and COS cells have been widely used as a heterologous system to determine the protease activity and substrate specificity of ROMs (13, 20, 21). Rhomboids perform a variety of diverse functions in different taxa ranging from control of growth to signaling and adhesion, etc., and this depends on cleavage of a wide range of unrelated substrates (17). Hence, rhomboids recognize the conserved feature of a less stable helical region in the transmembrane domains of otherwise dissimilar substrates. ROMs also show unusual kinetics that do not depend on substrate affinity but rather are driven by access of the substrate to the catalytic site (22). Collectively, these features suggest that ROMs have been coopted from an earlier quality control system for detecting unfolded membrane proteins (22).

The genomes of apicomplexan parasites encode multiple ROMs: for example, T. gondii has six and Plasmodium falciparum has eight ROM genes (23, 24). Of the six Toxoplasma ROMs, ROM6 is predicted to be a mitochondrial PARL-like ROM (14). The other five display stage-specific expression, with ROM1, ROM4, and ROM5 being expressed in tachyzoites; ROM4 in bradyzoites; and ROM1, ROM2, and ROM3 in sporozoites (13, 14). Given that many adhesins important for tachyzoite invasion, such as MIC2 and AMA1, are substrates for ROMs (13), it is of great interest to know the exact roles of the different ROMs expressed at the tachyzoite stage. Previous work has addressed the role of individual ROMs in T. gondii but has not established which, if any, of these proteases are essential. For example, T. gondii ROM1 (TgROM1) localizes to micronemes, and knockdown of TgROM1 resulted in a mild growth defect (25). TgROM4 is evenly distributed on the plasma membrane (13), and conditional suppression of this gene caused reduced adhesin shedding and decreased invasion (26). Additionally, overexpression of a dominant negative mutant of TgROM4 inhibited AMA1 cleavage and blocked parasite replication after invasion, suggesting signaling roles for AMA1 and TgROM4 (27). However, later studies did not find support for such roles, since a noncleavable mutant (28) and a complete knockout mutant (29) of AMA1 had normal intracellular replication, although they invaded less efficiently. TgROM5 also localizes to the surface but accumulates at the posterior end of the parasite membrane (13). In a heterologous system, TgROM5 displayed the highest activity against a broad range of substrates among all the T. gondii ROMs tested (13). Based on the posterior localization and robust protease activity, it was hypothesized that TgROM5 is the key ROM that cleaves surface adhesins during invasion to disengage adhesive interactions between the parasite and host cell receptors so that the parasite can fully enter the host cell (13).

In this study, we focused on the biological roles of the three ROMs (ROM1, ROM4, and ROM5) expressed in T. gondii tachyzoites. We generated gene knockouts and found that TgROM4 is the major tachyzoite ROM involved in surface adhesion processing and host cell invasion. Nonetheless, none of the ROMs were absolutely required for host cell penetration; however, intramembrane cleavage of micronemal adhesins was important for apical attachment and efficient host cell invasion by T. gondii.

RESULTS

Generation of ROM knockouts. Given its posterior localization and robust protease activity in a heterologous system, TgROM5 was proposed to be the key ROM shedding surface adhesins during invasion (13). Consistent with this, numerous attempts to knock out this gene by homologous replacement in the RH strain or its Δku80 derivative were not successful (data not shown). In order to obtain genetically modified strains to study the biological functions of TgROM5, we used the previously describedloxP-Cre technology to first flox the TgROM5 locus and subsequently delete this gene by expressing Cre recombinase (30, 31). The general design of this strategy is outlined in Fig. 1A. To flox the endogenous locus, a construct that contains the coding sequence of the corresponding ROM gene and the selection marker HXGPRT (hypoxanthine-xanthine-guanine phosphoribosyltransferase) cassette flanked by two loxP sites, as well as homologous arms for recombination, was transfected into the RH Δku80 Δhxgprt strain. After mycophenolic acid and xanthine selection, homologous replacement clones containing the floxed locus were identified by diagnostic PCR (Fig. 1A). To delete the floxed ROM gene, a plasmid (pmin-Cre-eGFP) (31) expressing the Cre recombinase was transiently transfected and selected with 6-thioxanthine (for HXGPRT deletion). Positive knockout clones were identified by diagnostic PCR and Western blotting (Fig. 1).

Using this system, we were able to efficiently generate clean TgROM5 knockouts (Δrom5) (Fig. 1C and D). The nonessential nature of TgROM5 made us suspect that it might have redundant functions with the other two tachyzoite ROM genes (i.e., TgROM1 and TgROM4). To check this possibility, and to determine the specific roles of each ROM, we used the loxP-Cre strategy and generated Δrom1, Δrom4, and Δrom5 single knockouts as well as double (Δrom1 Δrom4, Δrom1 Δrom5, and Δrom4 Δrom5) and triple (Δrom1 Δrom4 Δrom5) knockouts (Fig. 1B to H), which were confirmed by diagnostic PCR and Western blotting (for ROM4 and ROM5) (Fig. 1C to G). Δrom1 mutants were confirmed by additional PCRs to check the endogenous ROM1 gene (Fig. 1H) due to the lack of anti-TgROM1 antibody for Western blot analysis.

The fact that all the ROM mutants, including the triple knockout, were able to grow in tissue culture suggests that these enzymes are not essential for parasite survival in vitro. There are two other nonmitochondrial ROM genes (i.e., TgROM2 and TgROM3) that are not normally expressed at the tachyzoite stage (13), yet it remained possible that their expression was upregulated to compensate for the loss of ROM activity in our mutants. However, by quantitative real-time PCR (qRT-PCR), we detected very low levels of TgROM2 and TgROM3 transcripts in tachyzoites of the wild type (WT) and the triple knockout parasites, and there was no

September/October 2014 Volume 5 Issue 5 e01795-14

mbio.asm.org
FIG 1 Generation of ROM knockouts using the loxP-Cre technology. (A) Schematic illustration of the strategy used to generate the knockouts. First, a construct containing a ROM coding sequence and the HXGPRT selection marker flanked by two loxP sites was transfected into an RH ku80 strain to generate a floxed ROM strain. Subsequently, a Cre-expressing plasmid was transiently introduced into the floxed ROM strains to generate ROM knockouts by Cre-mediated recombination at the loxP sites. All the floxed ROM genes were hemagglutinin tagged at the N terminus, and ROM5 had an additional Ty tag at the C terminus. Diagnostic PCRs used to identify the correct clones are indicated based on the following amplicons. PCR1 and PCR2 checked for the proper (5' and 3', respectively) integration of the floxing construct into the corresponding ROM locus. PCR3 checked the replacement of endogenous ROM locus by the corresponding floxed ROM. Due to the removal of introns, the floxed locus gave a smaller PCR3 product than did the endogenous locus. PCR4 checked the junction of loxP after ROM deletion. (B) Diagram of the strains constructed in this study and the order of their construction. (C) Diagnostic PCRs on ROM5 deletion (ΔR5) and related strains. (D) Western blot analysis of ΔR5 and related strains using mouse anti-Ty or mouse anti-R5n (against the N terminus of... (Continued)
significant difference between the two strains (Fig. 11). These results indicate that TgROM2 and TgROM3 were not upregulated to compensate for the loss of ROM activities in the triple knockout. Collectively, these findings indicate that the three ROM genes expressed at the tachyzoite stage are not essential for parasite survival.

Growth of ROM knockouts in vitro and in vivo. To assess whether TgROM1, TgROM4, or TgROM5 played any role during parasite growth, we checked the ability of ROM knockout strains to form plaques on human foreskin fibroblast (HFF) monolayers. All of the ROM knockouts were able to produce plaques (Fig. 2A, B, and C), suggesting that none of the three ROM genes is essential for growth. Among repeated experiments, the \( \Delta\text{rom1} \Delta\text{rom4} \) triple knockout always produced fewer (40 to 60%) and smaller plaques (Fig. 2B and C) than did the WT strain. The \( \Delta\text{rom1} \Delta\text{rom4} \) double knockout also produced fewer (≈50%) plaques (Fig. 2B). Initially, we complemented \( \Delta\text{rom4} \) and \( \Delta\text{rom1} \Delta\text{rom4} \Delta\text{rom5} \) strains with TgROM4 at the \( \text{UPRT} \) (uracil phosphoribosyltransferase) locus, which is a nonessential gene. Although growth and invasion defects were rescued, the expression of ROM4 was lower than that in the wild type (data not shown). Therefore, we complemented \( \Delta\text{rom4} \) and \( \Delta\text{rom1} \Delta\text{rom4} \Delta\text{rom5} \) strains with TgROM4 at the endogenous locus to produce \( \Delta\text{rom4/ROM4} \) and \( \Delta\text{rom1} \Delta\text{rom4} \Delta\text{rom5/ROM4} \) strains, respectively (Fig. 1F; see also Fig. S1 in the supplemental material). Plaque

---

Figure Legend Continued

TgROM5, TgALD was detected by rabbit anti-ALD as a loading control. Li-Cor goat anti-mouse and -rabbit secondary antibodies were used to detect primary antibodies, and blots were scanned on the Li-Cor imaging system. (E) Diagnostic PCRs on ROM4 deletion (\( \Delta\text{rom4} \)) and related strains. (F) Western blot analysis of \( \Delta\text{rom4} \) and related strains using rabbit anti-R4n (against the N terminus of TgROM4). TgGRA7 was detected by mouse anti-GRA7 as a loading control. Blots were developed and scanned as described above. (G) Diagnostic PCRs on all ROM1 deletion (\( \Delta\text{rom1} \)) and related strains. (H) Additional PCRs confirming the loss of ROM1 sequence in \( \Delta\text{rom1} \) strains. (I) Analysis of \( TgROM2 \) and \( TgROM3 \) expression levels in wild-type (WT) and triple knockout \( \Delta\text{rom1} \Delta\text{rom4} \Delta\text{rom5} \) parasites by qRT-PCR. RNA isolated from unsporulated oocysts was used as a positive control, and the messenger levels of TgROM2 and TgROM3 were expressed as a percentage of actin in each sample. Means ± standard deviations of results from three independent experiments (\( n = 3 \)).
assays using these complemented lines indicated that TgROM4 was able to fully complement the growth defect of the triple knockout (Fig. 2A, B, and C). In an animal infection experiment, the survival of mice infected with ROM mutants was very similar to that of mice infected with WT parasites (Fig. 2D), indicating that these ROMs are not essential for parasite growth in vivo.

Taken together, these results suggest that while ROMs are important for optimal growth of tachyzoites in tissue culture, they are not absolutely essential for survival or virulence.

TgROM4 is important for efficient parasite motility and host cell invasion. To test the roles of ROMs in parasite motility, we used video microscopy to compare the motility patterns of WT and mutant parasites. When freshly egressed parasites were allowed to glide on a bovine serum albumin (BSA)-coated glass surface, Δrom4 mutants displayed significantly reduced helical gliding but increased twirling (Fig. 3A). This alteration was fully restored by TgROM4 complementation (Fig. 3A). Δrom1 or Δrom5 mutations (if not combined with Δrom4) did not cause any

FIG 3 Gliding motility, host cell invasion, and intracellular replication of WT and ROM mutant parasites. (A) Motility of freshly egressed tachyzoites on BSA-coated coverslips determined by time-lapse microscopy. Purified parasites were added to prewarmed Ringer’s buffer and imaged from 3 min at 1 frame/s. Means ± standard deviations from three independent experiments (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-way ANOVA was used to determine the overall difference (black asterisks) between each mutant and WT, and subsequent Bonferroni posttests (blue and green asterisks) were used to compare each motility type of the selected mutant to that of the WT. (B) Host cell invasion efficiencies determined by a two-color staining protocol that distinguishes intracellular from extracellular parasites. Representative data (means ± standard deviations) from three independent experiments (each with triplicates) with similar outcomes. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. Two-way ANOVA with Bonferroni posttests of mutants versus WT. (C) Moving junction formation during invasion determined by immunostaining. Naturally egressed parasites were used to challenge an HFF monolayer for 5 min at 37°C, and cells were immediately fixed for staining (see Fig. S2 in the supplemental material for details). Parasites falling into the indicated categories were counted and graphed as means ± standard deviations. Representative data (means ± standard deviations) from three independent experiments (each with triplicates) with similar outcomes. ***, P ≤ 0.001, two-way ANOVA with Bonferroni posttests. (D) Speed of invasion as determined by time-lapse video microscopy. Freshly purified parasites were used to invade HFF monolayers at 37°C and imaged for 3 min at 1 frame/s. The time spent during active penetration of each invaded parasite (from apical binding to complete internalization) was determined. Each dot represents one invasion event. Means ± standard deviations of results from two independent experiments (each with >3 replicates). (E) Intracellular replication of WT and the triple knockout parasites, which was determined by counting the number of parasites in each parasitophorous vacuole 18 h after invasion. Parasites were visualized by MAb DG52 staining of SAG1 followed by Alexa 488-conjugated goat anti-mouse secondary antibody. Means ± standard deviations (n = 6) of results from two independent experiments (each with triplicates).
obvious motility change (Fig. 3A), suggesting that TgROM4 is the primary tachyzoite ROM important for efficient gliding motility.

To test the role of ROMs in host cell invasion, we performed a two-color invasion assay that distinguishes extracellular parasites from intracellular parasites. Consistent with a previous report (26), TgROM4 was important for efficient invasion, as any mutant harboring Δrom4 displayed impaired invasion (Fig. 3B). In addition to reduced invasion, we also observed significantly more bound but noninvaded parasites with all Δrom4 strains (Fig. 3B), suggesting that increased nonproductive host cell attachment occurs in these mutants. Complementing with TgROM4 restored the invasion efficiency and reduced the attachment to normal levels (Fig. 3B), confirming that TgROM4 is responsible for these phenotypes. Deletion of TgROM1 or TgROM5, unless in combination with Δrom4, did not have any obvious effects on invasion efficiency or parasite attachment (Fig. 3B). The invasion phenotypes of the ROM deletion mutants matched well with their motility phenotypes. Taken together, these results suggest that TgROM4 plays a dominant role in parasite motility and host cell invasion, relative to other ROMs.

Initiation but not active penetration of invasion is impaired in Δrom4 parasites. Having demonstrated that Δrom4 mutants have reduced efficiency in host cell invasion, we wanted to further identify at what step of invasion they are impaired. First, we checked their ability to establish a MJ, a key step during invasion that initiates active penetration (32). Following a pulse invasion (5 min), the MJ was identified by the ring-like staining pattern of TgRON4 (see Fig. S2 in the supplemental material). With the WT strain, most parasites invaded successfully and did not display TgRON4 rings (classified as “invaded,” Fig. 3C), but instead they had a TgRON4 dot at the posterior end (see Fig. S2). A small portion of parasites were attached but did not show any TgRON4 staining (classified as “bound but no MJ,” Fig. 3C; see also Fig. S2), suggesting that they had not initiated invasion yet. Due to the high speed of invasion (see below), only a few parasites were observed in the process of invasion and they showed ring-shaped staining of TgRON4 (classified as “invading,” Fig. S2). In contrast, with the triple Δrom1 Δrom4 Δrom5 knockout, we observed fewer invaded parasites and significantly more parasites that were attached but failed to form an MJ (Fig. 3C). This pattern suggests that Δrom4 mutants engage in nonproductive attachment that does not lead to MJ formation, thus impeding invasion. These phenotypes were fully restored by TgROM4 complementation (Fig. 3C), indicating that they were caused by loss of ROM4.

Next, we looked at the speed of invasion, as determined by time-lapse video microscopy and expressed as the time required to penetrate a host cell (from apical binding to complete internalization). We compared the invasion speed of the Δrom1 Δrom4 Δrom5 triple knockout to that of WT parasites. Although triple knockout parasites invaded less frequently, those parasites that did invade penetrated the host cells at the same speed as did WT parasites (Fig. 3D). This finding suggests that ROMs likely do not play a role in the active penetration process.

Previous work using a dominant negative mutant of TgROM4 suggested that TgROM4 played a critical role in parasite replication by cleaving TgAMA1 to initiate replication after invasion (27). We reexamined this requirement using our ROM mutants but found that even the Δrom1 Δrom4 Δrom5 parasites replicated normally once they successfully invaded (Fig. 3E), indicating that these ROMs are not required for intracellular replication.

Cleavage of micronemal adhesins in ROM mutants. Next, we sought to determine the contribution of ROMs in processing micronemal adhesins such as MIC2 and AMA1, which are known substrates for ROMs (13, 28) and important for host cell infection (33, 34). We examined the release of the ectodomains of MIC2 and AMA1 into culture supernatant upon stimulation of microneme secretion. All mutants lacking ROM4 were completely defective in MIC2 processing (Fig. 4A), whereas Δrom1 or Δrom5 mutants (unless in combination with Δrom4) did not show any defects (Fig. 4A). The cleavage pattern of AMA1 in ROM mutants was very similar to that of MIC2, although overall shedding of AMA1 was much less efficient as seen by comparing the ratio of proteins in the supernatant to those in pellet (Fig. 4A). Similarly to MIC2, only Δrom4 mutants had dramatic reduction of AMA1 cleavage (Fig. 4A). For control purposes, we looked at the discharge of the nontransmembrane micronemal protein MIC5 (35), which is not a substrate for rhomboids. All the strains tested here secreted MIC5 at similar levels (Fig. 4A). Taken together, these findings indicate that none of the ROM mutants had defects in microneme secretion (i.e., release of adhesins from micronemes to the parasite surface) and that the reduced shedding of MIC2 and AMA1 into supernatant observed in Δrom4 mutants was due to the inhibition of intramembrane cleavage. These results also suggest that TgROM4 is the major tachyzoite ROM responsible for shedding of MIC adhesins, whereas the other two play minor roles in this process.

MIC2 was enriched on the parasite surface of Δrom4 mutants. To further check the inability of Δrom4 mutants to process MIC adhesins, we induced microneme secretion and subsequently quantified surface MIC2 levels by immunostaining and flow cytometry analysis. As expected, all mutants containing Δrom4 accumulated significantly more MIC2 on the surface than did WT parasites (Fig. 4B). In contrast, Δrom1 and Δrom5 (if not combined with Δrom4) mutants had surface MIC2 levels similar to that of WT parasites (Fig. 4B). In addition, surface MIC2 was almost completely shed in WT and other TgROM4-expressing parasites, since the signals for their surface MIC2 staining were very close to that of the negative control (Neg IgG, Fig. 4B). As a control, SAG1 staining was indistinguishable in all strains (WT and mutants) and was significantly higher than the control IgG staining (Fig. 4C).

We also visualized surface MIC2 by fluorescence microscopy after induction of microneme secretion. Consistent with the flow cytometry analysis, the levels of surface MIC2 on extracellular WT, Δrom1, and Δrom5 parasites were close to background, whereas those in Δrom4-containing mutants were greatly elevated (Fig. 4D). Together, these results indicate that ROM4 is needed to efficiently cleave MIC2 from the parasite surface.

Removal of micronemal adhesins from parasite surface during active invasion. Next, we sought to determine the fate of surface adhesins during active host cell invasion as monitored by quantitative immunofluorescence microscopy. Following induction of microneme secretion to release adhesins to the surface, parasites were allowed to invade host cells for 5 min and surface adhesin levels were measured by fluorescence microscopy. In both WT and Δrom1 Δrom4 Δrom5 parasites, AMA1 was evident on the surface of both extracellular and fully invaded parasites (Fig. 5A), consistent with the observation that it is not efficiently shed by extracellular parasites (Fig. 4A). In quantifying the fluorescent signals of invaded versus noninvaded parasites, we observed that
AMA1 levels on extracellular parasites were slightly higher than that of intracellular parasites in both strains (Fig. 5B). This is likely due to the difference of antibody accessibility, since similar differences were also observed for SAG1 levels (Fig. 5B). In contrast to AMA1, MIC2 was detected on extracellular triple mutant parasites but was undetectable on the surface of invaded parasites in both WT and the triple knockout strain (Fig. 5A). This was expected for WT cells, since the above-mentioned studies clearly demonstrated that MIC2 was almost completely cleaved from the surface by TgROM4 (Fig. 4B and D). However, extracellular Δrom1 Δrom4

FIG 4 Cleavage and surface accumulation of micronemal adhesins in ROM mutants. (A) Microneme secretion assay assessing the cleavage of transmembrane adhesins in ROM mutants. Microneme secretion was induced with 3% FBS and 1% ethanol, and the supernatants were separated from pelleted cells and subject to Western blot analysis. MIC2 and AMA1 were detected by monoclonal antibodies 6D10 and B3.90, respectively. TgALD was detected by rabbit anti-TgALD as a loading for pellet fractions and a cell lysis control for supernatant fractions. The nontransmembrane micronemal protein MIC5 detected with rabbit anti-TgMIC5 served as a microneme secretion control. Primary antibodies were visualized by Li-Cor secondary antibodies, and blots were developed on the Li-Cor imaging system. ecto, ectodomain; full, full length. (B and C) Accumulation of adhesins on parasite surface as determined by flow cytometry analysis. Following the induction of microneme secretion as in panel A, parasites were fixed and surface adhesins were stained with corresponding antibodies (6D10 for MIC2 in panel B and DG52 for SAG1 in panel C) and quantified by flow cytometry after Alexa 488-conjugated goat anti-mouse secondary antibody staining. WT parasites stained with mouse IgG (Neg IgG) were used as a negative control to set the gates. (D) Fluorescence microscopy examining surface protein levels. Samples were processed in the same way as in panel B but costained with 6D10 (followed by Alexa 488-conjugated secondary antibody, green channel) and rabbit anti-SAG1 (followed by Alexa 594-conjugated secondary antibody, red channel) for microscopic examination. Bar, 5 μm.
FIG 5  Fate of surface MIC2 during host cell invasion in WT and Δrom1 Δrom4 Δrom5 parasites. (A) Immunofluorescence staining of parasite surface antigens after host cell invasion. Parasites preinduced for microneme secretion were allowed to infect HFF monolayers for 5 min. Cells were then fixed, and extracellular parasites were stained with rabbit anti-SAG1. Following saponin permeabilization, parasites were stained with antibodies to AMA1 (MAb B3.90), MIC2 (MAb (Continued)
Δrom5 parasites accumulated MIC2 on the surface (Fig. 4B and D); therefore, the lack of detectable MIC2 on invaded mutant parasites was surprising. Quantification of fluorescent signals clearly showed that extracellular Δrom1 Δrom4 Δrom5 parasites exhibited much higher surface MIC2 levels than did intracellular Δwt parasites (Fig. 5B). However, once invaded, the two strains displayed similar (undetectable) levels of MIC2 on the surface (Fig. 5B), indicating that there is a ROM-independent mechanism to remove MIC2 from surface during invasion.

To more carefully track the removal of MIC2 relative to invading parasites in the triple knockout, we localized MIC2 and the MJ protein RON4 after a short invasion pulse (2 min). Interestingly, as soon as invasion started, the invaded portion of the parasite lacked any detectable MIC2, whereas the part behind the MJ (defined by the TgRON4 ring) was strongly stained (Fig. 5C). For parasites that were mostly inside the host cell, MIC2 staining was localized to a dot right behind the TgRON4 ring (Fig. 5C). After the parasite was fully internalized, the MIC2 dot was further separated from the TgRON4-containing structure (Fig. 5C). These results demonstrated that as soon as a parasite has passed through the MJ, the accumulated surface MIC2 is removed, by a yet-unknown mechanism.

The dot structure of MIC2 was frequently found to be in the vicinity of invaded Δrom1 Δrom4 Δrom5 parasites (yellow arrowheads, Fig. 5A), consistent with the accumulation of MIC2 at the site of host entry. To check the integrity of MIC2 molecules in these dots, we stained them with two antibodies, one for the ectodomain of MIC2 (MIC2ec) and the other for the cytoplasmic tail (MIC2t). Costaining with these two antibodies indicated that MIC2 dots contained full-length MIC2 protein since they were positively stained by both antibodies (Fig. 5D). In addition, we looked at the location of MIC2 dots relative to host cells by checking whether they were on the host cell surface or inside host cells. For this purpose, we stained them before and after saponin permeabilization with differently labeled MIC2ec antibodies. Interestingly, surface MIC2 on noninvaded parasites was easily detected before saponin treatment. However, MIC2 dots were detectable only after permeabilization (Fig. 5E), indicating that they were inside the host cell membrane.

**DISCUSSION**

Rhomboids are known to process apicomplexan transmembrane adhesins that are required for host cell recognition, suggesting that these proteases would also be important for host cell invasion (12, 13, 20, 36). However, because parasites express multiple ROMs with potentially overlapping substrate specificities (23, 24), the exact biological functions of individual proteases have been difficult to define. In the present study, we took a genetic approach to study the functions of ROM proteases in *T. gondii*. We generated single, double, and triple knockout mutants for the three ROM genes (ROM1, ROM4, and ROM5) expressed in *T. gondii* tachyzoites to assess their functions. Analysis of these mutants indicates that only ROM4 is required for efficient adhesin processing and host cell invasion, while ROM1 and ROM5 play negligible roles. Δrom4 mutants were unable to cleave adhesins like MIC2, which accumulated on the surface of extracellular parasites and led to impaired gliding motility, unproductive host cell attachment, and reduced invasion efficiency. However, mutants lacking individual or all three ROMs (i.e., the Δrom1 Δrom4 Δrom5 mutant) were viable, indicating that intramembrane cleavage of adhesins by ROM proteases is not essential. In addition, MIC2 that was secreted to the surface of Δrom4 mutants was still efficiently removed by a ROM-independent mechanism during active host cell entry. Taken together, these results demonstrate that, although not absolutely essential, ROM4 is the major tachyzoite ROM involved in cleaving micronemal adhesins on the parasite surface, where it serves to promote efficient apical attachment and facilitate invasion.

Proteolytic shedding of transmembrane micronemal adhesins from the membrane is a common feature of AMA1, MIC2, MIC6, MIC8, and MIC12 in *T. gondii* (8, 9, 37). Shedding is important for adhesin function, as shown by several previous studies. Mutations upstream of the transmembrane domain that block MIC2 shedding have a dominant negative effect, leading to increased host cell adhesion but reduced invasion (38). Similarly, mutations in the transmembrane domain that disrupt AMA1 shedding also decrease invasion efficiency (28). Shedding of these adhesins is mediated by intramembrane cleavage (10, 12, 28), suggesting the involvement of ROMs. Indeed, when assayed in a heterologous system, TgROM5 displayed robust ROM activity against a variety of substrates that included MIC2 and AMA1 (13, 28). However, the exact protease(s) that is responsible for adhesin shedding in *in vivo* and its biological substrate specificity are not known, partially due to the overlapping expression of multiple ROMs in *T. gondii* tachyzoites.

In the present study, we knocked out the three ROM genes (ROM1, ROM4, and ROM5) expressed in tachyzoites, individu-
ally and in combination. All mutants were viable in tissue culture and had acute virulence in mice similar to that of wild-type parasites at a dose of 200 parasites/mouse. It is possible that there might be appreciable differences in virulence of \( \Delta \text{rom} \) mutants in the type I background if a lower infection dose were used, or with a different strain background, such as less virulent type II strains. In addition, ROMs may play roles in other stages (such as bradyzoites) of the parasite’s life cycle, where ROM4 is highly expressed. We were not able to test the role of ROM4 in bradyzoites due to the intrinsic defect in differentiation of the RH strain; however, such phenotypes could be explored using type II strains, which readily differentiate in vitro and in vivo. Nonetheless, our analyses conducted in tachyzoites of a type I strain indicated that TgROM4 is the primary protease required for efficient AMA1 and MIC2 shedding. It was somewhat surprising to find that ROM4 was important for micronemal adhesin processing, whereas ROM5 did not play a major role in shedding, despite previous predictions that it would be essential for this process (13). This prediction was based on the fact that ROM5 localizes to the posterior end of the parasite surface and that it has the most robust activity against diverse substrates in a heterologous COS7 cell system (13). However, our results do not support a major role for ROM5 in \( T. \) gondii, since its deletion did not have any obvious adhesin processing or invasion phenotype. In contrast, although ROM4 was critical for adhesin shedding in \( T. \) gondii parasites, when expressed in COS7 cells it did not show any protease activity (13). The basis for this discrepancy between activity in COS7 cells and that in the parasite is currently unknown, but possible explanations include that (i) factors that regulate the substrate specificity and protease activity of ROMs may be unique to \( T. \) gondii and (ii) membrane composition and thickness may be different in COS7 versus \( T. \) gondii cells, which in turn may affect substrate processing by ROMs.

Although MIC2 was not processed by \( \Delta \text{rom}4 \) mutants, this adhesin was stripped off the surface and capped from the posterior end as the parasite moved through the MJ (Fig. 6), indicating the existence of a ROM-independent mechanism for adhesin removal during host cell penetration. The molecular nature of this mechanism is not yet known; however, it does not involve alternative proteolytic processing, as the protein was still full length after removal from the parasite surface. Removal of surface MIC2 may occur as a result of sieving at the MJ, since such a role has been proposed before to exclude host membrane proteins from the parasitophorous vacuolar membrane (39). In contrast, AMA1 traversed the MJ and largely remained on the surface of invaded parasites, suggesting that removal of surface adhesins is not a universal process. The retention of AMA1 on intracellular parasites, while MIC2 was almost completely removed, is also consistent with the differences in processing of these two substrates by extracellular parasites. Although the basis for this difference in processing is not known, it suggests that the defect in MIC2 processing contributes more to the phenotype of the \( \Delta \text{rom}4 \) mutants than

FIG 6 Model for MIC2 processing during invasion. Upon contact with host cells, parasites discharge their micronemal adhesins, including MIC2, to the surface from the apical end (step 1). MIC2 is then translocated to the posterior end by the motor complex (steps 1 to 3). In WT parasites (top), ROM4 is on the surface to cleave MIC2 to prevent aberrant host cell binding. Meanwhile, ROM4-mediated cleavage, along with the apical microneme secretion, allows MIC2 to form a temporal apical-to-posterior gradient that helps the parasite to reorientate and establish an MJ (step 1). Once MJ is formed, microneme secretion stops and surface MIC2 is shed by ROM4 (steps 2 and 3). The parasite starts to penetrate into the host cell (step 3), and no MIC2 is on the surface when it is fully internalized (step 4). TgROM4-deficient parasites (bottom) are not able to cleave surface MIC2. They lose the MIC2 gradient and often bind to host cells laterally (step 5); such nonproductive binding inhibits MJ formation and reduces invasion efficiency. However, if the mutant parasite does orientate correctly and form an MJ (step 6), it invades the host cell at normal speed (step 7). In addition, the unprocessed MIC2 that is accumulated on the surface is removed during active penetration and is capped behind the MJ (step 7). After its full entry, capped MIC2 is separated from the parasite (orange arrowhead) and accumulates within the host cell (step 8).
does the defect in AMA1 processing. Consistent with this, T. gondii mutants of AMA1 that are refractory to rhomboid processing have only a modest effect on invasion and do not cause significant nonproductive adhesion as the Δrom4 mutants (28).

Proteolytic shedding of surface adhesins is a common feature in apicomplexan parasites; however, there are differences between species in the complexity and essentiality of this pathway. Unlike T. gondii, alternative adhesin processing seems to be common in Plasmodium. For example, thrombospondin-related anonymous protein (TRAP), a MIC2 homolog in Plasmodium berghei, is mainly cleaved by ROMs within the membrane but can also be cleaved by another protease outside the membrane, albeit with lower efficiency (40). Similarly, shedding of AMA1 in P. falciparum is mediated predominantly by SUB2 at a juxtamembrane site, but it can also be processed by ROMs within the membrane (41). The existence of alternative processing pathways in Plasmodium spp. implies that adhesin shedding is more important in these parasites than in T. gondii. Consistent with this, a noncleavable TRAP mutant of P. berghei resulted in nonmotile and noninfectious sporozoites (40), and shedding-resistant mutants of EBA175 and AMA1 could not be stably introduced into the genome of P. falciparum (36, 41). Finally, unlike the situation in T. gondii, where none of the tachyzoite-stage ROMs was essential, four out of the eight P. berghei ROM genes were refractory to gene deletion in the asexual blood stage (42). Included in these putatively essential ROMs is P. berghei ROM4 (PbROM4), which is the orthologue of TgROM4, highlighting key differences in the essentiality of these pathways between these two parasites.

Our studies demonstrate that in T. gondii, ROM4 is the primary rhomboid protease required for efficient adhesin processing, and knockout mutants studied here behaved similarly to the previously described ROM4 conditional knockdown mutant (26) and the noncleavable MIC2 mutants (38). All three mutants lead to accumulation of unprocessed adhesins on the surface, resulting in unproductive host cell attachment and reduced invasion. Collectively, these data are consistent with the following model for adhesin shedding and function of ROM4 during invasion (Fig. 6).

Materials and Methods

Parasite strains and growth conditions. The RH Δku80 Δhxgprt strain (45) was used to generate all the genetically modified lines used in this study. All strains were maintained as tachyzoites by growth on monolayers of human foreskin fibroblasts (HFFs) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and 20 μg/ml gentamicin. Parasites used in experiments were freshly purified with 3-μm Nucleopore filters to remove host cell debris, as described previously (26).

Generating constructs to flox the ROM loci. Plasmids generated and used in this study are listed in Table S1 in the supplemental material. All ROM floxing constructs contain three fragments that were ligated together by LR reactions of corresponding entry vectors generated by BP cloning (Life Technologies, Carlsbad, CA). The fragments include the 5′ untranslated region (UTR) and 3′ UTR of each ROM gene surrounding a middle fragment containing loxP sites that flank the ROM coding sequence and the selectable marker HXGPRT (Fig. 1A contains an illustration). The 5′ UTR and 3′ UTR of each ROM gene were amplified from RH genomic DNA (using primers listed in Table S2 in the supplemental material) and cloned into pDONR-P4P1r and pDONR-P2P3r, respectively. The middle fragment of each ROM gene was amplified (using primers listed in Table S2) from templates indicated in Table S1 and cloned into pDONR221 by BP cloning. The template vectors containing the middle fragments of ROM genes were generated as follows. For ROM1, the HXGPR selection cassette obtained from pUNIV-KI by Spel digestion was inserted into pSIHA9-ROM1 (25) at the SpeI site to give the plasmid pHA-ROM1/HXGPRT. For ROM4, the HA-ROM4 coding sequence was amplified (primers listed in Table S2) from pSIHA9-ROM4 (26) and cloned into pUNIV-KI. For ROM5, exon 1 of TgROM5 was amplified from RH genomic DNA (using primers listed in Table S2) and cloned into pUNIV-KI between the NcoI and EcoRI sites. Subsequently, the rest of the ROM5 coding sequence was amplified (primers indicated in Table S2) from a previously published HA-ROM5 construct (13) and cloned into pUNIV-KI-Exon 1 at the EcoRI site.

Generation of ROM knockout and complementing strains. To generate transgenic parasites with floxed ROM loci, the floxing plasmids were digested using restriction endonucleases (pDEST-loxP-HAR1 was digested with PstI and PciI to generate a 7.4-kb fragment) and cloned into pS1HA9-ROM1 (25) at the NdeI site to give the plasmid pDEST-loxP-HAR1. For ROM4, the HA-ROM4 construct (13) and cloned into pHA-ROM4/HXGPRT. For ROM5, the HA-ROM5 construct (13) was digested with NdeI and PstI to generate a 6.7-kb fragment, and pDEST-loxP-HAR4 was digested using restriction endonucleases (pDEST-loxP-HAR4 was digested with SpeI to generate a 5.5-kb fragment, pDEST-loxP-R5Ty was digested with BglII and PciI to generate a 7.4-kb fragment) and cloned into pS1HA9-ROM4 (26) and cloned into pHA-ROM4/HXGPRT. For ROM5, the HA-ROM5 construct (13) was digested with NdeI and PstI to generate a 6.7-kb fragment, and pDEST-loxP-HAR4 was digested using restriction endonucleases (pDEST-loxP-HAR4 was digested with SpeI to generate a 5.5-kb fragment, pDEST-loxP-R5Ty was digested with BglII and PciI to generate a 7.4-kb fragment) and cloned into pUNIV-KI. To knock out the ROM genes surrounding a floxing plasmid pmin-Cre-eGFP (31) was transiently introduced into the RH floxed strains by electroporation and selected the loss of HXGPRT with 200 μg/ml 6-thioxanthine. ROM knockout clones were identified by PCR that confirmed the junction of loxP after ROM removal. The expression of ROMs in the floxed and deletion strains was further confirmed by Western blotting using antibodies raised from the N termini of ROM4 and ROM5, respectively (see below). The deletion of ROM1 was confirmed by two additional PCRs (PCR5 and PCR6; primers listed in Table S2 in the supplemental material) to detect the endogenous ROM1 gene.

To complement ROM4 at the endogenous locus, the ROM4 floxing construct digested with NdeI and PstI was transfected into Δrom4 and Δrom1 Δrom4 Δrom5 strains and selected with 25 μg/ml mycophenolic acid and 50 μg/ml xanthine (Sigma-Aldrich, St. Louis, MO). Single-cell clones were obtained by limiting dilution. Positive clones were identified by diagnostic PCR using primers listed in Table S2 in the supplemental material.

To knock out the ROM genes in the floxed strains, the Cre recombinase-expressing plasmid pmin-Cre-eGFP (31) was transiently introduced into the RH floxed strains by electroproporation and selected the loss of HXGPRT with 200 μg/ml 6-thioxanthine. ROM knockout clones were identified by PCR that confirmed the junction of loxP after ROM removal. The expression of ROMs in the floxed and deletion strains was further confirmed by Western blotting using antibodies raised from the N termini of ROM4 and ROM5, respectively (see below). The deletion of ROM1 was confirmed by two additional PCRs (PCR5 and PCR6; primers listed in Table S2 in the supplemental material) to detect the endogenous ROM1 gene.

To complement ROM4 at the endogenous locus, the ROM4 floxing construct digested with NdeI and PstI was transfected into Δrom4 and Δrom1 Δrom4 Δrom5 strains and selected with 25 μg/ml mycophenolic acid and 50 μg/ml xanthine. After limiting dilution, positive clones were identified by diagnostic PCR as described above, and ROM4 expression was confirmed by Western blotting.

September/October 2014 Volume 5 Issue 5 e01795-14

mbio.asm.org

mbio.asm.org  on October 25, 2014 - Published by mbio.asm.org
Production of antibodies against TgROM4 and TgROM5. Recombinant proteins corresponding to the N termini of TgROM4 (R4n, containing residues V25 to G240 of TgROM4) and TgROM5 (R5n, containing residues M1 to R319 of TgROM5) were expressed and purified from BL21(DE3)/pLysS containing pET28a-R4n and pET16b-R5n (plasmids listed in Table S1 in the supplemental material), respectively. A New Zealand White rabbit (Govance, Princeton, NJ) (R4n) or CD-1 mice (Charles River Laboratories, Wilmington, MA) (R5n) were injected with purified antigens and boosted two more times after initial injection. Subsequently, the animals were sacrificed and sera were collected for analysis.

Plaque assay. Purified parasites were used to infect fresh host cell monolayers seeded in 6-well plates and grown for 7 days without movement. Cells were then fixed with 70% ethanol and stained with 0.1% crystal violet. Plaques were scanned using an Epson scanner and analyzed as previously reported (47). Experiments were repeated three times independently, and each strain was assayed in triplicate within each experiment.

Virulence assay. Freshly egressed parasites were intraperitoneally injected into CD-1 female mice (200 parasites/mouse and 5 mice/strain). The survival of mice was monitored, and data were analyzed as previously described (48).

Invasion assay. A previously established two-color staining protocol (26) that distinguished extracellular and intracellular parasites was used to determine the host cell invasion efficiencies of the ROM mutants, with the following modifications: the invasion assay was done using host cells seeded in 96-well plates, and after staining, the plates were imaged on the Cytation 3 cell imaging multimode reader (BioTek, Winooski, VT). Data were analyzed with Gen5 software (BioTek, Winooski, VT). Experiments were repeated three times independently (each with triplicates).

Time-lapse microscopy assay of parasite motility and invasion. To visualize parasite motility, freshly egressed tachyzoites were purified and resuspended in intracellular buffer (5 mM NaCl, 142 mM KCl, 2 mM EGTA, 1 mM MgCl₂, 5.6 mM glucose, 25 mM HEPES-KOH, pH 7.2) (49) before analysis. Motility was induced by adding parasites to BSA-coated glass-bottom culture dishes (MatTek, Ashland, MA) that contained Ringler’s buffer (155 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, pH 7.2), as previously described (26). Parasites were then kept at 37°C with Tempcontrol 37-2 (Carl Zeiss, Inc., Thornwood, NY) and imaged on the Zeiss Axio Observer Z1 time-lapse imaging system (Carl Zeiss, Inc., Thornwood, NY) under X40 magnification for 3 min at 1 frame/s. Motility patterns were analyzed as previously described (26).

To measure the speed of invasion, freshly purified parasites were resuspended in invasion medium (DMEM, 20 mM HEPES, 3% FBS, pH 7.4), used to infect HFF host cells seeded on glass-bottom culture dishes, and imaged as described above. The time of each invasion event (from apical binding to complete internalization) was used to estimate invasion speed. Each strain was analyzed in two independent experiments, and each experiment contained 20 to 30 invasion events taken from 4 to 6 movies.

Intracellular replication assay. Replication of indicated strains within host cells was determined as previously described (47). After SAG1 staining, the number of vacuoles containing the indicated number of parasites (i.e., 1, 2, 4, or 8 cells) was counted from ≥100 vacuoles from three separate coverslips per experiment. Two independent experiments were conducted, and combined results were graphed.

Microneme secretion assay. Microneme secretion was monitored based on the release of adhesins into culture supernatants as previously described (26). In brief, purified tachyzoites (10⁶ in 100 µl) were incubated with 3% FBS plus 1% ethanol in invasion medium at 37°C for 15 min. Subsequently, supernatants were separated from pellet cells by centrifugation (400 x g for 10 min), and the proteins of interest in each fraction were analyzed by Western blotting. Samples from each strain (25% of the supernatant fractions and 10% of the pellet fractions) were resolved on 10% SDS-PAGE gels and analyzed by Western blotting. MIC2 and AMA1 were detected by monoclonal antibodies (Mabs) 6D10 (9) and B3.90 (50), respectively. TgALD was detected by rabbit anti-TgALD (51), and MIC5 was detected with rabbit anti-TgMIC5 (52). Primary antibodies were detected with Li-Cor secondary antibodies (Life Technologies, Carlsbad, CA), and blots were developed on the Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Moving junction formation. Freshly isolated parasites were allowed to invade HFF monolayers in invasion medium at 37°C for 5 min. Samples were then immediately fixed with 4% parafomaldehyde, stained, and examined by epifluorescence microscopy as previously described (26). Each strain was tested in three independent experiments, each with triplicates.

Quantification of surface adhesin levels on parasites. Freshly egressed parasites of the WT and Δrom1 Δrom4 Δrom5 strains were treated with 3% FBS plus 1% ethanol to induce their microneme secretion for 15 min in invasion medium. Subsequently, they were allowed to invade HFF monolayers for 5 min, fixed with 4% paraformaldehyde, and subjected to immunostaining. Samples were first stained with rabbit anti-SAG1 followed by Alexa 594-conjugated goat anti-rabbit secondary antibody. Following 0.1% saponin permeabilization, samples were stained with MAb 6D10 (for MIC2), MAB B3.90 (for AMA1), or MAb DG52 (for SAG1) and visualized by Alexa 488-conjugated goat anti-mouse secondary antibody. Parasite and host nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss Axioskop 2 MOT Plus microscope equipped with a 63× numerical-aperture (NA)–1.6 oil immersion lens and an AxioCam MRm Monochrome camera (Carl Zeiss, Inc., Thornwood, NY). Fluorescent signals were quantified by the Volocity software (PerkinElmer, Waltham, MA). Two independent experiments (each with triplicates) were conducted.

Distribution of surface MIC2 during invasion. Freshly purified parasites were used for microneme secretion as described above and subsequently allowed to invade HFF monolayers for 2 min at 37°C. Parasites were immediately fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. MIC2 was detected by MAb 6D10 followed by Alexa 488-conjugated goat anti-mouse secondary antibody, and RON4 was visualized by rabbit anti-TgRON4 followed by Alexa 594-conjugated goat anti-rabbit secondary antibody. Images were acquired as described above.

Flow cytometry and fluorescence microscopy of surface MIC2. Purified parasites were induced for microneme secretion as described above and subsequently fixed with 4% paraformaldehyde for immunostaining. MIC2 was stained with MAb 6D10. SAG1 was detected by MAb DG52, and ChromPure mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as a negative control. Primary antibodies were stained with Alexa 488-conjugated goat anti-mouse secondary antibody. Samples were analyzed by flow cytometry using FACSCanto (Becton, Dickinson, Franklin Lakes, NJ) as previously described (26).

To visualize MIC2 by fluorescence microscopy, parasites were prepared in the same way as that described above but co-stained with rabbit anti-SAG1 and MAb 6D10 and examined by epifluorescence microscopy as described above.

RT-PCR. Total RNA from parasites was extracted with the RNeasy kit (Qiagen Inc., Valencia, CA), and 400 ng RNA was subjected to reverse transcription using SuperScript III reverse transcriptase according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Subsequently, quantitative PCR was performed using the SYBR green master mix (Life Technologies, Carlsbad, CA) containing corresponding primer pairs (see Table S1 in the supplemental material) as previously described (26). Total RNA isolated from unsporulated oocysts (provided by John Boothroyd) was used as a positive control for TgROM2 or TgROM3 expression. Three independent experiments were performed, and combined results were reported.

Statistics. Statistical comparisons were conducted in Prism (GraphPad Software, Inc., La Jolla, CA) using one-way analysis of variance (ANOVA) and Bonferroni’s multiple comparison posttests. For comparing two groups that contain multiple data sets, two-way ANOVA with...
Bonferroni multiple comparison posttests was used. Significant differences across all repeated experiments are indicated.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01795-14/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.5 MB.
Table S1, PDF file, 0.03 MB.

ACKNOWLEDGMENTS
We are grateful to Gary Ward, Vern Carruthers, and John Boothroyd for providing antibodies; Ke Hu for the Cre-expressing plasmid; members of the Sibley lab and Dominique Soldati-Favre for helpful discussions; and Jennifer Banks and Qiuiling Wang for technical assistance.

The work was supported by a grant from the NIH (AI034036).

REFERENCES
29. Mital J, Meissner M, Soldati D, Ward GE. 2005. Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demon-


