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# Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion

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## Abstract

Apicomplexan parasites secrete transmembrane (TM) adhesive proteins as part of the process leading to host cell attachment and invasion. These microneme proteins are cleaved in their TM domains by an unidentified protease termed microneme protein protease 1 (MPP1). The cleavage site sequence (IA↓GG), mapped in the *Toxoplasma gondii* microneme proteins TgMIC2 and TgMIC6, is conserved in microneme proteins of other apicomplexans including *Plasmodium* species. We report here the characterisation of novel *T. gondii* proteins belonging to the rhomboid family of intramembrane-cleaving serine proteases. *T. gondii* possesses six genes encoding rhomboid-like proteins. Four are localised along the secretory pathway and therefore constitute possible candidates for MPP1 activity. *Toxoplasma* rhomboids TgROM1, TgROM2 and TgROM5 cleave the TM domain of *Drosophila* Spitz, an established substrate for rhomboids from several species, demonstrating that they are active proteases. In addition, TgROM2 cleaves chimeric proteins that contain the TM domains of TgMIC2 and TgMIC12.

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**Keywords:** Apicomplexa; Intramembrane; Cleavage; Rhomboid; Serine; Protease; *Toxoplasma gondii*

## 1. Introduction

*Toxoplasma gondii* belongs to the phylum Apicomplexa which is composed of a large number of protozoan parasites that cause severe diseases in a wide variety of hosts, including humans, farm and household animals. The most notorious member is *Plasmodium falciparum*, the etiologic agent of malaria, which is responsible for millions of deaths every year. *T. gondii* causes toxoplasmosis, a disease which can be fatal in immunocompromised patients and may lead to severe congenital defects if pregnant women develop a primary infection. *T. gondii* and other apicomplexans

invade host cells by an active process dependent on the actomyosin system of the parasite (Dobrowolski and Sibley, 1996; Dobrowolski et al., 1997; Meissner et al., 2002). Invasion also requires the sequential secretion of the contents of membrane-bound organelles called micronemes and rhoptries (Carruthers and Sibley, 1997). Micronemes discharge diverse adhesive proteins that bind to host cells (reviewed in Carruthers, 2002; Dowse and Soldati, 2004) and redistribute towards the posterior pole of the parasite during invasion (Carruthers et al., 1999). Among these microneme proteins, the members of the thrombospondin-related anonymous protein (TRAP) family, including TgMIC2 in *T. gondii*, are known to play an essential role in host cell invasion (Sultan et al., 1997; Yuda et al., 1999; Templeton et al., 2000; Huynh et al., 2003). In *T. gondii*, microneme proteins form complexes, such as TgMIC1/MIC4/MIC6, TgMIC3/MIC8 and TgMIC2/M2AP (Soldati et al., 2001; Carruthers, 2002; Dowse and Soldati, 2004), which are composed of a transmembrane escorter protein (TgMIC6, TgMIC8 or TgMIC2) essential for the correct

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targeting of the complex to the micronemes, and soluble proteins, some of which exhibit host cell binding properties (TgMIC1, TgMIC4 and TgMIC3) (Fourmaux et al., 1996; Garcia-Reguet et al., 2000; Brecht et al., 2001).

After discharge by the micronemes, TgMIC2 is processed by two proteolytic activities named microneme protein protease 1 and 2 (MPP1 and MPP2, respectively) (Carruthers et al., 2000). Microneme protein protease 1 cleavage results in the release of the ectodomain of TgMIC2 from the parasite surface, an event that is critical for efficient invasion (Brossier et al., 2003). The release of TgMIC2 was previously shown to be sensitive to the serine protease inhibitor 3,4-dichloroisocoumarin (DCI) (Carruthers et al., 2000). 3,4-Dichloroisocoumarin may act directly or indirectly on MPP1. The C-terminal MPP1 cleavage also occurs on several other transmembrane proteins such as TgMIC6, TgMIC12, TgMIC8 (Reiss et al., 2001) and TgAMA-1 (Donahue et al., 2000) as well as members of the TRAP family in *Plasmodium* and other apicomplexan species. The cleavage site of TgMIC6 by MPP1 was recently mapped by mass spectrometry, and shown to be within the transmembrane (TM) domain at the site IA↓GG, a sequence conserved in several apicomplexan transmembrane microneme proteins (Opitz et al., 2002). A recent study confirmed that TgMIC2 is also cleaved at this conserved intramembrane site (Zhou et al., 2004). Additionally, a lysine situated 11 residues upstream from the TM domain of TgMIC2 was shown to be required for MPP1 mediated cleavage and successful invasion (Brossier et al., 2003). Taken together, these findings suggest that MPP1 activity is most likely essential and ubiquitous throughout the phylum of Apicomplexa.

Several groups have suggested that a rhomboid-like protease may be responsible for MPP1 activity (Urban and Freeman, 2003; Dowse and Soldati, 2004; Kim, 2004; Sibley, 2004). Rhomboids form a family of polytopic membrane proteases conserved throughout evolution and found in bacteria, yeast, plants and animals (Koonin et al., 2003). The founding member of the family, *Drosophila* Rhomboid-1 was characterised as an intramembrane serine protease, which cleaves the epidermal growth factor (EGF)-like substrates Spitz, Gurken and Keren, resulting in the secretion of the soluble factors from the cell (Lee et al., 2001; Urban et al., 2001, 2002a). Rhomboids are the only known intramembrane proteases of the serine class, and are inhibited by the serine protease inhibitor DCI (Urban et al., 2001). More recent studies have established that members of this large family of proteases are involved in controlling processes other than intercellular signalling, such as mitochondrial membrane fusion (Esser et al., 2002; Herlan et al., 2003; McQuibban et al., 2003), and new substrates have been identified for mammalian rhomboids (Lohi et al., 2004; Pascall and Brown, 2004). Some rhomboids exhibit broad substrate specificity, recognising helix-destabilising residues in the luminal region of a TM domain and cleaving diverse substrates, including

transmembrane adhesins of *T. gondii* (Urban and Freeman, 2003).

Rhomboid-like proteins have recently been identified in the genome of *P. falciparum* (Wu et al., 2003), and we report here the characterisation of TgROM1, TgROM2, TgROM4, and TgROM5, four members of a family of six rhomboid-like proteins in *T. gondii*.

## 2. Materials and methods

### 2.1. General reagents

Restriction enzymes were purchased from New England Biolabs and Invitrogen. The secondary antibodies for Western blotting were from Biorad or Amersham Biosciences, and for immunofluorescence were from Molecular Probes. Reverse-transcriptase-PCR (RT-PCR) amplifications were performed using the Titan One-Tube RT-PCR kit (Roche). All PCR reactions were performed with either the GeneAmp High Fidelity PCR system or AmpliTaq (Applied Biosystems).

### 2.2. Parasite strains

*T. gondii* tachyzoites (RH strain wild-type and RH $\Delta$ gprt<sup>−</sup>) were grown in human foreskin fibroblasts (HFF) maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 25 µg/ml gentamicin. Parasites were harvested after complete lysis of the host cells and purified by passage through 3.0 µm filters and centrifugation in PBS.

### 2.3. Cloning of the *T. gondii* rhomboid genes

Plasmids pT8mycTgROM1, pT8mycTgROM2 and pT8mycTgROM4 and pT8mycTgROM5 were all constructed by cloning the corresponding cDNA between the *Nsi*I and the *Pac*I sites in T8MycGFP-HX (Hettmann et al., 2000). Total RNA was isolated from freshly released parasites using TRIzol (Invitrogen). The cDNAs for each gene were amplified by RT-PCR according to the manufacturer's instructions, using the Titan one tube RT-PCR system (Roche) and the primers listed in Table 1. The plasmid pT8TgROM1Ty was generated by cloning the TgROM1 cDNA between the *Eco*R I and *Nsi* I sites in the pT8MLCTy-HX vector (Herm-Gotz et al., 2002).

Plasmids pHA-TgROM1, pHA-TgROM2, pHA-TgROM4 and pHA-TgROM5 were prepared by cloning the appropriate cDNA between the *Bam*H I and *Eco*R I sites on the pCAN-HA2 vector, a mammalian expression vector based on pcDNA3 that adds an N-terminal hemagglutinin (HA) tag. The plasmids pHA-Rho1 and pHA-RHBDL2 carry inserts encoding *Drosophila* Rhomboid-1 and human RHBDL2 respectively.

Table 1  
Primers used in this study

Primer name	5'-3' Sequence	Restriction sites added:	To clone into:
TgROM1-1	CCGGGATC- CAATGGCGCGGGTCC- GAACACTCGC	<i>Bam</i> HI	pCAN- HA2
TgROM1-2	GCCGAATTCT- CATTTGCTGCA- CAAACTGACGCATG	<i>Eco</i> RI	pCAN- HA2
TgROM1-8	CCAATG- CATGCGCGGGTCC- GAACACTCGCAG	<i>Nsi</i> I	pT8myc
TgROM1-9	CCAATG- CATTTTTGCTGCA- CAAACTGACGCATG	<i>Nsi</i> I	pT8myc
TgROM1-12	CCGGAATTCCTTTT- CGA- CAAAATGGCGCGGGT- CCGAACAC	<i>Eco</i> RI	pT8Ty1
TgROM1-13	GGCATGCATGAATG- TAGGA- CAAATCCCTGTGGCA	<i>Eco</i> RI	pT8Ty1
TgROM2-1	GGCATGCATAAG- GATCCCGCCAA- CATTCGGACGTTGTCG	<i>Nsi</i> I/ <i>Bam</i> HI	pT8myc pCAN HA2
TgROM2-2	CCGTAAATTA- GAATTCGCATAACTTC- GAATGCGCTTGCT- GAGG	<i>Eco</i> RI/ <i>Pac</i> I	pT8myc pCAN- HA2
TgROM3-1	GGCATGCATAAG- GATCCCGCTTCGTCTG- GTGGATCAGATGTG	<i>Nsi</i> I/ <i>Bam</i> HI	pT8myc pCAN- HA2
TgROM3-2	CCGTAAATTA- GAATTCGGGAGG- TAGTCGGACATCCGAG	<i>Eco</i> RI/ <i>Pac</i> I	pT8myc pCAN- HA2
TgROM4-8	GGCATGCATAAA- GATCTCGTGTG- GACTTCGGCCGTCG	<i>Nsi</i> I/ <i>Bgl</i> II	pT8myc pCAN- HA2
TgROM4-10	GGAATTCCTTAAT- TAAGGTTCAAGATAA- TACTGCGCATCC	<i>Eco</i> RI/ <i>Pac</i> I	pT8myc pCAN- HA2
TgROM5-10	GGCATGCATAAA- GATCTGTCGTC- GAAAGGTG- GATCTTCTC	<i>Nsi</i> I/ <i>Bgl</i> II	pT8myc pCAN- HA2
TgROM5-11	GCCCAATTGTTAAT- TAAT- TATTGGCCTGCCCTCG- TCTGC	<i>Mfe</i> I/ <i>Pac</i> I	pT8myc pCAN- HA2

#### 2.4. Parasite transfection (*RHxgprt*<sup>−</sup>) and selection of stable transformants

Transient transfections were undertaken by electroporation as previously described (Soldati and Boothroyd, 1993). Stable transformants were selected for by hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) expression in the presence of mycophenolic acid and xanthine as described earlier (Donald et al., 1996). Parasites were cloned by limiting dilution in 96 well plates and analysed for the expression of the transgenes by indirect immunofluorescence assay (IFA).

#### 2.5. Cell fractionation and Western blot analysis of *T. gondii* tachyzoites

Crude extracts, or pellet and soluble fractions from freeze-thaw lysis of *T. gondii* tachyzoites were subjected to SDS-PAGE as described previously (Soldati et al., 1998). Western blot analysis was carried out using on 10% polyacrylamide gels run under reducing conditions and the samples that were probed for rhomboid proteins were not boiled prior to loading. Proteins were transferred to Hybond ECL nitrocellulose. For detection, the membranes were incubated with primary antibodies (anti-myc, 1:1,000; anti-Ty-1, 1:1,000; anti-HA, 1:500) diluted in PBS, 0.05% Tween 20, 5% skimmed milk, and then with affinity-purified horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:3,000) and bound antibodies visualised using the ECL system (Amersham).

#### 2.6. Indirect immunofluorescence confocal microscopy

All manipulations were carried out at room temperature. Intracellular parasites grown in HFF on glass slides were fixed with 4% paraformaldehyde for 20 min. Following fixation, slides were rinsed in PBS-0.1 M glycine. Cells were then permeabilised in PBS, 0.2% Triton-X-100 for 20 min and blocked in the same buffer with 2% BSA. Slides were incubated for 60 min with primary antibodies diluted in blocking solution, washed and incubated for 60 min with Alexa 594 goat anti-rabbit or Alexa 488 goat anti-mouse antibodies (Molecular Probes), diluted 1:3,000 in blocking solution. Slides were mounted in Vectashield and kept at 4 °C in the dark. 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. All other micrographs were obtained with a Zeiss Axiophot with a camera (Photometrics Type CH-250). Adobe Photoshop (Adobe Systems, Mountain View, CA) was used for image processing.

#### 2.7. Mammalian cell culture and transfections

HEK-293T cells were maintained in DMEM containing 100 i.u. Penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal calf serum. Cells for transfection were seeded on 35-mm culture dishes (Nunc) that had been pretreated with poly-L-lysine. After 24 h, the cultures were transfected with plasmid DNA using Lipofectamine (Invitrogen) as described (Pascall et al., 2002). Cells were transfected with DNA constructs encoding a myc-tagged substrate protein (0.5 µg) and Star (a protein required for Spitz trafficking; 0.5 µg), with or without a HA-tagged rhomboid (*Drosophila* Rhomboid-1 (0.1 µg) or human RHBDL2 (0.1 µg) or a TgROM (0.5 µg). Plasmid pcDNA3 was included to maintain the total input DNA at 2 µg. Cells

and medium were harvested for Western blot analysis of immunoreactive proteins approximately 24 h after the start of transfection as described (Pascall and Brown, 2004).

### 3. Results

#### 3.1. *Toxoplasma gondii* possesses six rhomboid-like proteases

An agreement for the nomenclature of apicomplexan rhomboids has been made between our groups and others (Dowse and Soldati, 2005). Six genes coding for rhomboid-like proteins (ROMs) have been found in *T. gondii*, and are named TgROM1–6. The cDNAs corresponding to TgROM1, TgROM2, TgROM4 and TgROM5 were amplified from *T. gondii* tachyzoite total RNA by RT-PCR, cloned and sequenced. All attempts to amplify the cDNA for TgROM3 failed, suggesting that this gene is not transcribed in the tachyzoite stage. Supporting this view, all ESTs specific for the TgROM3 gene are found in cDNA prepared from unsporulated oocysts while none are present in the abundant tachyzoite EST database (<http://www.cbil.upenn.edu/paradbs-servlet/>). TgROM6 exhibits a predicted mitochondrial targeting signal at the N-terminus, and clusters with other mitochondrial rhomboids in a phylogenetic analysis (Dowse and Soldati, 2005), thus it is very likely fulfilling a function unrelated to MPP1.

Rhomboids are conserved in apicomplexan parasites, some being of similar size to *Drosophila* Rhomboid-1, and others being significantly larger. The amino acid sequence alignment of some of the smaller and larger ROMs from *T. gondii*, *P. falciparum*, *Plasmodium berghei* and *Eimeria tenella* are shown in Fig. 1(A) and (B), respectively. All *T. gondii* sequences contain six to eight putative TM domains as predicted by TMPred, ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

The apicomplexan ROMs contain many of the critical conserved residues shown to be required for *Drosophila* Rhomboid-1 activity. Urban et al. (2001) demonstrated that mutations of W151, R152, N169, G215, S217 (the putative catalytic serine) and H281 abolished Rhomboid-1 activity. The putative catalytic serine in most rhomboid-like proteases is found within the sequence GASG (Koonin et al., 2003). In the smaller apicomplexan ROMs, the sequence GAST is found, whereas in the larger ROMs the sequence GSSG is present and conserved. Residues corresponding to N169 and H281, the other two residues proposed to comprise the catalytic triad of *Drosophila* Rhomboid-1 (Urban et al., 2001), are conserved in all apicomplexan ROMs (Fig. 1(A) and (B)). Another characteristic of rhomboid-like proteins that is also found in the apicomplexan ROMs is the presence of very short loops between the predicted TM domains, with the exception of the loop between the first and second TM domains.

In addition, the larger apicomplexan ROMs contain a second longer loop between the sixth and seventh TM domains and also have longer N and C termini than the smaller rhomboids (Fig. 1(B)).

#### 3.2. Subcellular distribution of the *T. gondii* ROM proteases

Epitope-tagging each cDNA and expressing the transgenes in *T. gondii* under control of the tubulin-1 promoter allowed examination by immunofluorescence microscopy of the subcellular localisations of TgROM1, TgROM2, TgROM4, and TgROM5. TgROM1 tagged either at the N-terminus (myc) or at the C-terminus (Ty-1) localised to a compartment at the apical end of the parasite (Fig. 2(A) and results not shown), suggesting a microneme localisation based on the predominant colocalisation with the microneme marker TgMIC4. Additional labelling in the late secretory pathway is possibly an effect of protein overexpression as a similar distribution with a significant accumulation in the secretory pathway was previously observed with some microneme proteins (Reiss et al., 2001). Since N- and C-terminal tagging of TgROM1 resulted in the same localisation, the other ROMs were tagged only with an N-terminal myc-epitope. mycTgROM2 showed a focused staining at the apical side of the nucleus, typical of Golgi staining in *T. gondii* (Pelletier et al., 2002). Double immunofluorescence analysis with the Golgi marker GRASP-YFP (Pelletier et al., 2002), suggests that TgROM2 accumulates in a distinct region of the Golgi stack from GRASP-YFP, likely to correspond to the *trans*-Golgi network (TGN) (Fig. 2(B)). Both mycTgROM4 and mycTgROM5 localised to the parasite pellicle, potentially the plasma membrane (Fig. 2(C) and (D)). When mycTgROM5 is overexpressed, it is localised to the surface, although the signal is not as homogenous as for mycTgROM4. Furthermore, unidentified internal structures are labelled as well as residual bodies, which form in the parasitophorous vacuole during parasite division. TgMIC4 is present in the interior of these structures, which are indicative of the parasite poorly tolerating the overexpression of ROM5. Western blot analysis of the parasites expressing TgROM1Ty and mycTgROM4 showed that the tagged proteins are of expected size and separate into the membrane fraction (Fig. 3).

#### 3.3. TgROM1, TgROM2 and TgROM5 are active proteases

The TM domain of the *Drosophila* Spitz protein is cleaved by some, but not all, rhomboids from a variety of organisms (Urban et al., 2001, 2002b; Urban and Freeman, 2003). However, the efficiency of release of the cleaved product depends on where processing occurs in the cell. *Drosophila* Rhomboid-1 cleaves Spitz in the Golgi and soluble Spitz is efficiently released from cells (Urban et al., 2001, 2002a). However, when Rhomboid-1 is artificially retained in the ER by adding a KDEL retrieval signal to its



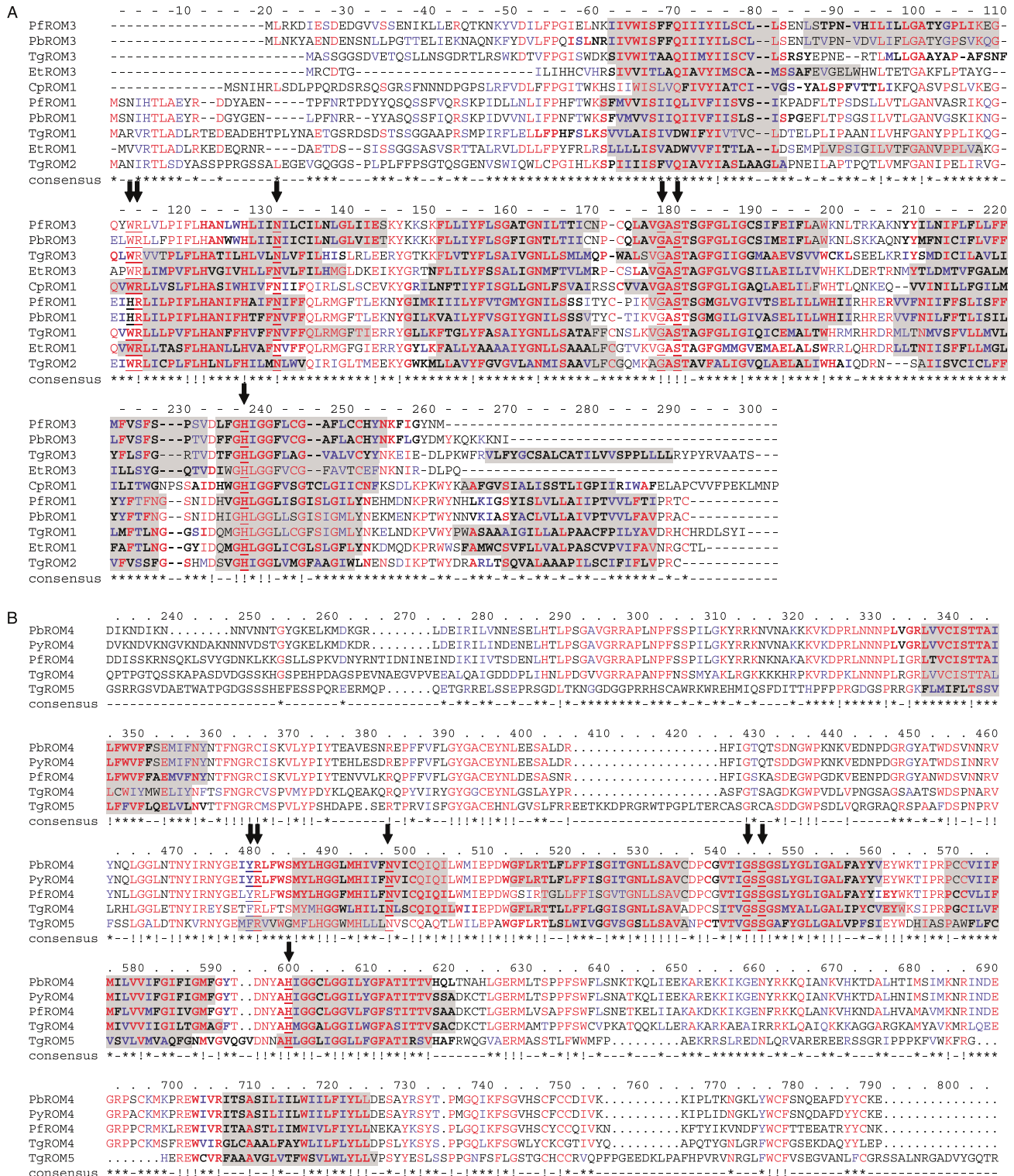


Fig. 1. Sequence analysis of *Toxoplasma gondii* rhomboid-like protein (ROM) sequences. (A) Alignment of the shorter ROMs from some apicomplexan species (TgROM1, AAT29065; TgROM2, AAT29066; TgROM3, AAT39987; PFROM1, PF11\_0150; PFROM3, MAL8P1.16; PbRom1, Pb\_82b04plc; PbROM3, Pb\_213e08plc; CpROM1, CplOWA\_III\_s2; EtROM1, Contig877; EtROM3, Contig7050). Red residues: 40% conserved or identical. Blue residues: 40% similar. (B) Alignments of the longer ROMs from some apicomplexan species, excluding the divergent N and C termini. (TgROM4, AAT29067; TgROM5, AAT47708; PFROM4, PFE0340c; PbROM4, Pb\_256f11plc; PyROM4, PY04351). In both alignments TMpred predicted transmembrane (TM) domains are highlighted in grey, TMHMM predicted TM domains are in bold letters. Residues corresponding to W151, R152, N169, G215, S217 and H218 of *Drosophila* Rhomboid-1 are underlined and marked with arrows. Consensus line: \* denotes conserved or similar residues, ! denotes identical residues.

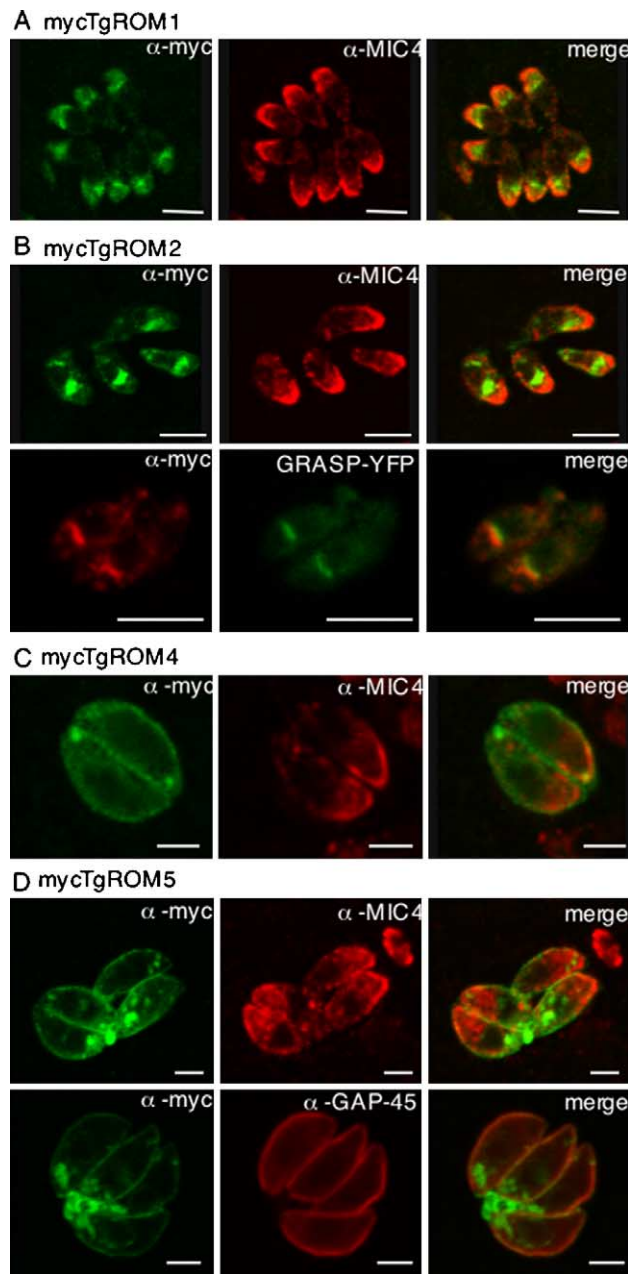


Fig. 2. Subcellular localisation of the *Toxoplasma gondii* rhomboid-like proteins (ROMs) by epitope tagging (N-terminal myc tag) in tachyzoites. (A) Predominantly micronemal localisation of TgROM1; anti-myc ( $\alpha$ -myc) in green, and anti-MIC4 ( $\alpha$ -MIC4, micronemal marker) in red. Scale bars 5  $\mu$ m. (B) Golgi-localisation of TgROM2;  $\alpha$ -myc in green or red (upper and lower panel, respectively),  $\alpha$ -MIC4 in red and the *cis*-Golgi marker GRASP-YFP in green. Scale bars 5  $\mu$ m. (C) Peripheral localisation of TgROM4 at the parasite plasma membrane;  $\alpha$ -myc in green and  $\alpha$ -MIC4 in red. Scale bars 2  $\mu$ m. (D) Peripheral localisation of TgROM5 at the plasma membrane and in undefined intracellular structures and residual bodies sometimes produced after parasite division;  $\alpha$ -myc in green,  $\alpha$ -MIC4 or  $\alpha$ -GAP45 in red. Scale bars 2  $\mu$ m.

C-terminus, the release of ER-cleaved Spitz is inefficient (Urban et al., 2002a). Furthermore, several bacterial rhomboids cleave the Spitz TM domain when the proteins are co-expressed in COS cells but, in some cases, little if any

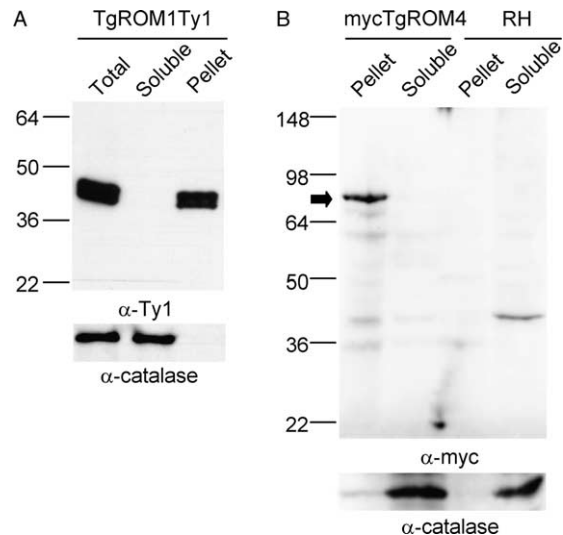


Fig. 3. Western blot of recombinant epitope-tagged TgROM1 and 4 in *Toxoplasma gondii*, probing total cell lysate and soluble and pellet fractions. (A) Anti-Ty1 ( $\alpha$ -Ty1) clearly detects TgROM1Ty1 in the cell lysate and the pellet but not in the soluble fraction. (B) Anti-myc ( $\alpha$ -myc) clearly detects mycTgROM4 (arrow) in the pellet but not in the soluble fraction. In both panels, anti catalase ( $\alpha$ -catalase) demonstrates that the cells have been efficiently lysed, as it is present in the soluble fraction, and only minimal traces are in the pellet.

cleaved product is released, suggesting that these rhomboids may be retained in the ER of mammalian cells (Urban et al., 2002b). Consequently, to establish whether a particular rhomboid can cleave Spitz, it is necessary to look for the presence of a cleaved product in cell lysates as well as in medium samples. However, the detection of a cleaved intracellular product is complicated by the complex protein band pattern that results from the extensive glycosylation of Spitz (Schweitzer et al., 1995; Urban et al., 2001, 2002a). To circumvent this problem, we generated myc-tagged Spitz from which amino acids 31–77 had been deleted (named  $\Delta$ Spitz), thereby removing one potential N-glycosylation site and 21 possible O-glycosylation sites.

To determine whether TgROMs are able to cleave the TM domain of Spitz, HEK293T cells were co-transfected with constructs encoding myc-tagged  $\Delta$ Spitz together with HA-tagged TgROMs. In Western Blots, HA-tagged TgROM1 and TgROM5 resolve as single bands of 31 kDa and >97 kDa apparent molecular weight, respectively, whereas TgROM2 appears as a series of bands (24–31 kDa and 41 kDa) (compare Fig. 4(A)). The abundance of this last band is markedly increased on heating the sample prior to electrophoresis (results not shown), and may be due to aggregation of the protein. We have been unable to demonstrate expression of HA-tagged TgROM4 in HEK293T cells (not shown). *Drosophila* Rhomboid-1 and human RHBDL2, which are known to cleave the Spitz TM domain (Urban et al., 2001), were used as positive controls in these experiments and caused the appearance of a 32 kDa myc-reactive protein, cleaved  $\Delta$ Spitz, that was detectable both intracellularly (arrowhead in Fig. 4(B)) as well as in

the medium (arrow in Fig. 4(C)). This protein was not present in cells expressing  $\Delta$ Spitz alone (Fig. 4(B)) or in cells expressing an inactive RHBDL2 mutant (results not shown), suggesting that the 32 kDa protein is cleaved intracellular  $\Delta$ Spitz. Consistent with this, an immunoreactive protein of this size was present in the medium of cells expressing *Drosophila* Rhomboid-1 or RHBDL2 (Fig. 4(C)). The medium also contains smaller myc-reactive proteins that appear not to be present in cell lysates and may be generated by proteolysis of the 32 kDa protein after its release from cells. The expression of TgROM1, TgROM2 or TgROM5 led to the cleavage of  $\Delta$ Spitz as indicated by the appearance of an intracellular 32 kDa protein, although the effect of TgROM2 was small (Fig. 4(B)). In contrast to the action of *Drosophila* Rhomboid-1 or RHBDL2, the cleaved product was barely detectable in the medium of cells expressing either TgROM1 or TgROM2 suggesting that in HEK293T cells these *Toxoplasma* rhomboids are located in the ER and not in the Golgi apparatus or a later secretory compartment. However, in the presence of TgROM5, the cleaved Spitz product was also found in the culture medium, which suggests that TgROM5 is

localised in the Golgi apparatus or later in the secretory pathway of HEK293T cells. These results demonstrate that TgROM1, TgROM2 and TgROM5 are active proteases that can cleave the Spitz TM domain.

### 3.4. TgROM2 cleaves microneme protein transmembrane domains

In order to test the ability of *Toxoplasma* rhomboids to cleave the TM domains of microneme proteins, we replaced the TM and cytoplasmic domains of  $\Delta$ Spitz with the corresponding regions of MIC2 or MIC12 to generate the chimeric proteins  $\Delta$ Spi/MIC2 and  $\Delta$ Spi/MIC12 respectively. *Drosophila* Rhomboid-1 and human RHBDL2 were used as positive controls in the assay, as they have previously been demonstrated to cleave the TM domains of MIC2 and MIC12 in synthetic constructs (Urban and Freeman, 2003). In agreement with this, *Drosophila* Rhomboid-1 and human RHBDL2 both cleaved the  $\Delta$ Spi/MIC2 and  $\Delta$ Spi/MIC12 chimeric substrates efficiently, resulting in both an intracellular and a secreted cleavage product (Fig. 5). In contrast, co-expression of either TgROM1 or TgROM5 with the chimeric substrates failed to generate any detectable cleaved fragments in either the cell lysates or in the medium, indicating that, at least in the context of the chimeric constructs used, these proteases cannot cleave the TM domains of MIC2 or MIC12. In the presence of TgROM2, however,  $\Delta$ Spi/MIC12 was cleaved to generate an intracellular fragment, although release of the cleaved product into the medium was not detected. In addition, generation of an intracellular fragment from  $\Delta$ Spi/MIC2 was also detectable, although this cleavage was very weak (Fig. 5). However for neither substrate was the cleavage catalysed by TgROM2 as efficient as that by *Drosophila* Rhomboid-1 or RHBDL2 (Fig. 5).

## 4. Discussion

We report here the characterisation of members of the rhomboid family of proteases in the parasite *T. gondii*. Homologues of these genes are also present in other apicomplexans including *Plasmodium*. All these proteins described here contain multiple predicted membrane-spanning domains and conserve residues that have been implicated in the catalytic activity of rhomboid-like serine proteases. The six genes identified in *T. gondii* code for three smaller rhomboids, two larger rhomboids, and one putative mitochondrial rhomboid. Apart from TgROM6, expressed sequence tags (ESTs) from different life stages of *T. gondii* corresponding to each gene are present in the ToxoDB database with the exception of TgROM3 for which only ESTs from unsporulated oocysts can be found (Li et al., 2003) (Table 2). Our failure to amplify the cDNA of TgROM3 by RT-PCR from tachyzoite total RNA preparations reinforces the view that this gene is

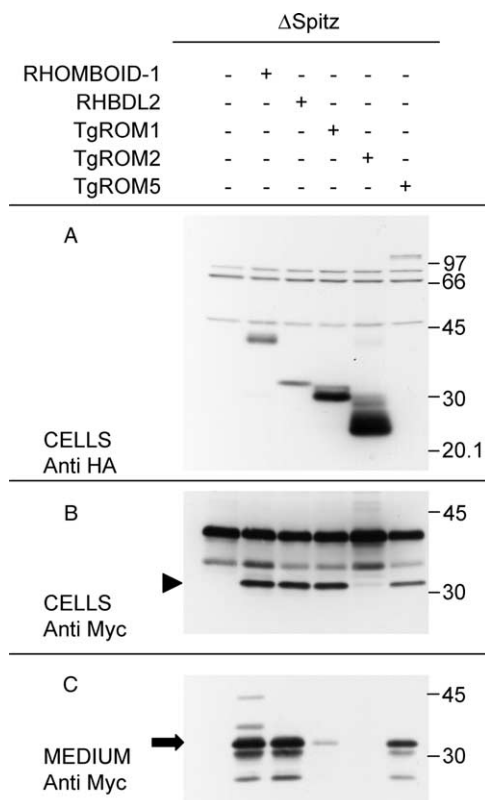


Fig. 4. TgROMs cleave the Spitz TM domain. HEK293T cells were transfected with myc-tagged  $\Delta$ Spitz and HA-tagged rhomboids as described in Section 2.7. (A) Western blot of cell lysates using anti-HA to detect rhomboid protein expression. (B) Western blot of cell lysates using anti-myc to detect the  $\Delta$ Spitz substrate and the cleaved  $\Delta$ Spitz product (arrowhead). (C) Western blot of samples of culture medium using anti-myc to detect the release of the cleaved  $\Delta$ Spitz product (arrow) from cells. All size markers denote kDa.



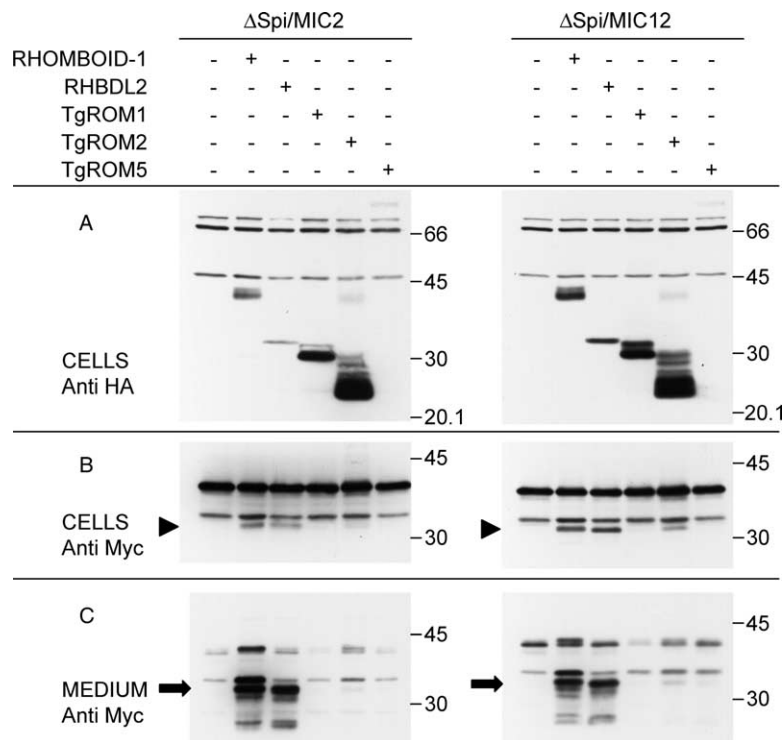


Fig. 5. TgROM2 can use MIC2 and MIC12 TM domains as a substrate. HEK293T cells were transfected with myc-tagged  $\Delta$ Spi/MIC2 or  $\Delta$ Spi/MIC12 and HA-tagged rhomboids as described in the Section 2. (A) Western blot of cell lysates using anti-HA to detect rhomboid protein expression. (B) Western blot of cell lysates using anti-myc to detect the cleavage products (arrowheads) of the chimeric  $\Delta$ Spi/MIC2 or  $\Delta$ Spi/MIC12 substrate proteins. (C) Western blot of samples of culture medium using anti-myc to detect the release of the cleaved  $\Delta$ Spi/MIC2 or  $\Delta$ Spi/MIC12 products (arrows) from cells. All size markers denote kDa.

developmentally regulated. In *P. berghei*, preliminary results based on RT-PCR analysis suggest that expression of PbROM3, the apparent homologue of TgROM3, appears to be restricted to the gametocyte stage (Dowse, unpublished data). As MPP1 is expressed in tachyzoites, and possibly in all invasive stages of the parasite including bradyzoites and sporozoites, the absence of TgROM3 transcripts in tachyzoites excludes TgROM3 as a candidate for MPP1. As MPP1 activity has previously been monitored constitutively at the parasite cell surface (Opitz et al., 2002), the intracellular localisation of the remaining TgROMs was considered to be key to determining which if any are likely to correspond to this protease. By this criterion TgROM4 and TgROM5 are possible candidates, as they show immunocytochemical localisation at the parasite cell surface.

In a mammalian cell culture assay system, we have demonstrated that TgROM1, TgROM2 and TgROM5 are active proteases that are capable of cleaving the TM domain of the widely used rhomboid substrate, *Drosophila* Spitz. However, of these, only TgROM2 shows any activity towards chimeric proteins containing the TM and cytoplasmic domains of MIC2 and MIC12, although we cannot dismiss the possibility that TgROM1 and/or TgROM5 would cleave the full-length MIC proteins. Therefore, in this context, only TgROM2 appears to be a potential candidate for MPP1. However definitive conclusion on the cleavage of

$\Delta$ Spi/MIC constructs by TgROM2 would require additional controls using an inactivated mutant form of the protease. As the cellular localisation of TgROM2 is not that which is expected for MPP1 the only obvious remaining rhomboid candidate is TgROM4. Unfortunately, our attempts to express TgROM4 in mammalian cell cultures have been unsuccessful and, consequently, we cannot currently determine whether it is able to cleave the TM domains of the MIC proteins.

Rhomboid-like proteins have recently been implicated as candidates for MPP1 based on several lines of evidence (Urban and Freeman, 2003; Dowse and Soldati, 2004; Kim, 2004; Sibley, 2004): (i) TgMIC2 and TgMIC6 cleavage by MPP1 occurs within their TM domains, close to the luminal/extracellular end, and rhomboids are the only known intramembrane proteases to cleave near to the luminal face of their substrates' TM domains resulting in secretion/release; (ii) rhomboids are the only known intramembrane proteases shown not to require a prior cleavage event on their substrates for their activity; (iii) rhomboids are the only known intramembrane proteases of the serine class and are sensitive to DCI which is the only inhibitor previously shown to block MMP1-dependent processing of TgMIC2; and (iv) rhomboid-1 from *Drosophila* and human RHBDL2 have previously been shown to cleave MIC2, MIC6 and MIC12 TM domains (Urban and Freeman, 2003). Our study now adds three important lines of evidence to support

Table 2  
Summary of *Toxoplasma gondii* rhomboids

	Accession number	Stage of expression (ESTs)	Subcellular localisation	Cleavage of Spitz	Cleavage of MICs
TgROM1	AY596191	Tachyzoite, Bradyzoite	Micronemes	++	–
TgROM2	AY596192	Tachyzoite, Sporulated oocyst	Golgi	+	+
TgROM3	AY587209	Sporulated oocyst	?	nd	Nd
TgROM4	AY596193	Tachyzoite, Bradyzoite	Plasma membrane	Not expressed	Not expressed
TgROM5	AY587208	Tachyzoite	Plasma membrane	++	–
TgROM6	TgTwin-Scan_4036	?	Predicted mitochondrion	nd	Nd

this hypothesis: (i) rhomboid-like proteases are present in *T. gondii* and conserved throughout the Apicomplexa, as expected of MPP1; (ii) two rhomboid-like proteins, TgROM4 and TgROM5, are localised to the parasite surface, as expected of MPP1; and (iii) three *T. gondii* rhomboids have been demonstrated to be active, one of which can use TgMIC2 and TgMIC12 TM domains as substrates. However, on the basis of these results, we are unable to identify one of the *Toxoplasma* rhomboid proteases as MPP1. Of significant interest is that an examination of the repertoire of rhomboids proteins in the currently published apicomplexan genomes also provides evidence for the identification of MPP1. This survey revealed that the apicomplexan parasites contain between three and eight genes coding for putative rhomboid proteases although some of them are very exotic and might not be active proteases. All Apicomplexa including *Cryptosporidium* and *Theileria* species have at least one stage of their life cycle which moves by gliding. Consequently, they all possess at least one or more members of the TRAP family of microneme proteins, many of which harbor the conserved cleavage site IA↓GG. Based on phylogenetic analysis, only one rhomboid is commonly present and conserved in all apicomplexan species and corresponds to TgROM4, supporting it as a plausible candidate for MPP1 (Dowse and Soldati, 2005).

The definitive assignment of MPP1 to a rhomboid-like protease will require an analysis of the biological actions of these rhomboids, which will be made possible by conditional disruption of the ROM genes. The identification of apicomplexan proteases that belong to the invasion machinery will represent a critical step for the development of novel therapeutic strategies to cure the diseases caused by this important group of pathogens.

A recent study of the *Toxoplasma* rhomboids demonstrated the cleavage of full length MIC2, and synthetic constructs containing MIC6 and MIC12 TM domains, by TgROM5. It was also shown that TgROM5 redistributes to the posterior of the parasite upon microneme secretion. This led to the conclusion that TgROM5 is responsible for MPP1 activity (Brossier et al., 2005). The discrepancy between this study and the results reported here might be explained by the use of different cell lines and constructs for the cleavage assays.

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