A family of transmembrane microneme proteins of *Toxoplasma gondii* contain EGF-like domains and function as escorters

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Summary

TgMIC6, TgMIC7, TgMIC8 and TgMIC9 are members of a novel family of transmembrane proteins localized in the micronemes of the protozoan parasite *Toxoplasma gondii*. These proteins contain multiple epidermal growth factor-like domains, a putative transmembrane spanning domain and a short cytoplasmic tail. Sorting signals to the micronemes are encoded in this short tail. We established previously that TgMIC6 serves as an escorter for two soluble adhesins, TgMIC1 and TgMIC4. Here, we present the characterization of TgMIC6 and three additional members of this family, TgMIC7, -8 and -9. Consistent with having sorting signals localized in its C-terminal tail, TgMIC6 exhibits a classical type I membrane topology during its transport along the secretory pathway and during storage in the micronemes. TgMIC6 is processed at the N-terminus, probably in the trans-Golgi network, and the cleavage site has been precisely mapped. Additionally, like other members of the thrombospondin-related anonymous protein family, TgMIC2, TgMIC6 and TgMIC8 are proteolytically cleaved near their C-terminal domain upon discharge by micronemes. We also provide evidence that TgMIC8 escorts another recently described soluble adhesin, TgMIC3. This suggests that the existence of microneme protein complexes is not an exception but rather the rule. *TgMIC6* and *TgMIC8* are expressed in the rapidly dividing tachyzoites, while *TgMIC7* and *TgMIC9* genes are predominantly expressed in bradyzoites, where they presumably also serve as escorters.

Key words: Apicomplexa, *Toxoplasma gondii*, Micronemes, Epidermal growth factor-like domain, Secretion, Processing, Escorter

Introduction

T. gondii belongs to the phylum Apicomplexa, which includes important pathogens of human and animals. These obligate intracellular parasites share many common structural and functional features, most notably the phylum-defining apical complex, which consists of cytoskeletal elements and secretory organelles. *T. gondii* actively invades a broad range of cells within its mammalian host by a mechanism distinct from phagocytosis that depends on the ability of parasites to glide. Gliding locomotion involves the parasite actomyosin system, the apical release and subsequent redistribution of adhesins toward the posterior pole of the parasite. Micronemes are specialized apical secretory organelles, presumed to play a predominant role in the early phase of the invasion process by discharging adhesins capable of interacting with host cell receptors. Apicomplexan microneme proteins belonging to the TRAP family (Thrombospondin related anonymous protein) exhibit a transmembrane spanning domain and provide directly or indirectly, a molecular anchor for parasite gliding via their cytoplasmic tail (Kappe et al., 1999). TgMIC2, a close homologue of TRAP, and TgAMA1 are the only transmembrane micronemal proteins of *T. gondii* characterized to date (Donahue et al., 2000; Hehl et al., 2000).

To determine if additional, potentially paralogous, transmembrane proteins exist, we searched the Toxoplasma expressed sequence tag (EST) database (Ajioka, 1998) and found ESTs with significant similarity to the transmembrane and cytoplasmic tail domains of TRAP. This database proved to be a valuable resource for the identifying genes/proteins phylogenetically restricted to the Apicomplexa that may be crucial for the establishment of intracellular parasitism (Ajioka et al., 1998). The cloning of the first of these novel genes, *TgMIC6*, revealed the presence of three epidermal growth factor (EGF)-like domains in the extracellular domain of the molecule. Such domains were previously identified on proteins anchored by lipids at the surface of *Plasmodium* ookinetes and merozoites (Kaslow et al., 1988; Blackman et al., 1991). Additional searches led to the identification of three more

micronemal proteins, which are structurally similar to TgMIC6 and thus constitute a novel family of transmembrane proteins containing multiple EGF-like domains. We have localized these proteins to the micronemes and studied their processing and patterns of expression during stage differentiation of *T. gondii*. Two of these members, TgMIC6 and TgMIC8, are expressed in the tachyzoite stage and function as escorters, targeting soluble adhesins to the micronemes.

Materials and Methods

Strains and plasmids

T. gondii tachyzoites (RH strain wild-type and RH*hxgprt*⁻) were grown in human foreskin fibroblasts (HFF) or in Vero cells (African green monkey kidney cells) maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 25 µg/ml gentamicin. The bacterial strains for recombinant DNA techniques were *Escherichia coli* XL1-Blue and XLOR. *E. coli* BL21(DE3) was used for recombinant protein expression. The helper phage, ExAssist from Stratagene, was used for the in vivo excision of the phagemid vectors from the λZAPII clones. Restriction enzymes were purchased from New England Biolabs.

Toxoplasma cDNA library screening

Complete cDNA clones of the *TgMIC6* gene were obtained by screening the RH (EP) λ cDNA expression library in λZAPII from NIH AIDS reagent repository. The non-radioactive labeling of nucleic acids based on the digoxigenin system from Boehringer Mannheim was used for the screening. A DIG-dUTP-labeled PCR DNA fragment amplified from one EST clone (Ctoxqual2_2870) was used as the probe. The detection of positive clones was achieved by chemiluminecsence with CPDS according to the manufacturer (Roche). The cDNA corresponding to TgMIC7, MIC8 and MIC9 were amplified by RT-PCR and sequenced. Analysis of the DNA and protein sequences was performed using programs available on the NCBI web site and Expasy server (http://www.expasy.ch/tools). Prediction of signal peptide cleavage sites and transmembrane spanning domains have been obtain using the software at: http://www.cbs.dtu.dk/services/SignalP/index.html and http://www.biokemi.su.se/~server/toppred2/toppredServer.cgi

Toxoplasma gDNA library screening

Cosmid clones containing the *TgMIC6, MIC7 and MIC8* loci were isolated from a cosmid library. The library was made in a SuperCos vector modified with SAG1/ble *T. gondii* selection cassette inserted into its *Hin*dIII site. The library was prepared from a *Sau*3AI partial digestion of RH genomic DNA ligated into the *Bam*HI cloning site and was kindly provided by D. Sibley and D. Howe. Probes were labeled using DIG-11-dUTP. The hybridization and chemiluminescent CSPD® detection were carried according to the manufacturer (Roche).

DNA and RNA preparations and semi-quantitative RT-PCR

Parasites were harvested after complete lysis of the host cells and purified by passage through 3.0 µm filters and centrifugation in PBS. Genomic DNA was isolated from purified parasites by sodium dodecyl sulfate/proteinase K lysis followed by phenol/chloroform, chloroform extractions and ethanol precipitation (Sibley and Boothroyd, 1992). Total RNAs were prepared using RNA clean from AGS GmbH according to the manufacturer. mRNA expression levels were measured by semi-quantitiative RT-PCR as previously described (Yahiaoui et al., 1999). *T. gondii* cysts were isolated from mice chronically infected with tachyzoites of the 76K strain for 2 months. In vivo bradyzoites were freed by pepsin digestion (0.05 mg/ml pepsin in 170 mM NaCl, 60 mM HCl) for 5-10 minutes at 37°C. These in vivo bradyzoites and tachyzoites cultivated in HFF were lysed with 1% SDS, 50 mM sodium acetate pH 5.2, 10 mM EDTA and total RNA was isolated following two phenol extractions at 65°C and ethanol precipitation. For controls, total RNA from uninfected brain cells of mice and HFF were also isolated. For reverse transcriptase PCR, total RNA was digested with DNase and checked by PCR to confirm that no DNA remained in these samples before reverse transcription was done. Semi-quantitative RT-PCR was performed by using tenfold serial dilutions of tachyzoite and bradyzoite cDNAs. The cDNA products were amplified with 50 pmol of each primer and 2.5 U of AmpliTaq DNA polymerase (Promega) in 50 µl reaction volumes (5 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.01 M EDTA, 0.1 mM DTT, 5% glycerol, 0.1% Triton X-100, 1.5 mM MgCl$_2$ and 200 µM dNTP). Thermal cycling conditions were: (1) denaturation at 94°C for 10 minutes; (2) denaturation at 94° C for 1 minute followed by the annealing at 50-60°C (depending of each pair primer); (3) elongation for 3 minutes at 72°C; (4) at the end, a 10 minutes at 72°C additional extension was done. Usually, 30-35 cycles were performed. Primers used in this experiment were as follows: the *T. gondii* housekeeping α-tubulin gene (Nagel and Boothroyd, 1988) 5′-ATGAGAGAG-GTTATCAGCATC-3′ and 5′-T-TAGTACTCGTCACCATAGCC-3′.

TgMIC2   5′-CGGAATTCGCCAGTTACCACTACTATTTGA-3′ and 5′-CGGAATTCAGCTCCAGTGTCGGATCCCCAT-3′.

TgMIC4   5′-TGCATGCATTTCTGCGATTTTGGCGGTAGC-3′ and 5′-CCTTAATTAAACTGCAGCTTCTGTGTCTTTCGCTTC-3′.

TgMIC6 5′-CGGAATTCGACTCAACAGAACCGGGGA-3′ and 5′-CGGAATTCCAATGTTTTGGCTAATCC-3′.

TgMIC7 5′-CGGGATCCTTCGAGTGCAGATGTAATGAATAC-CGG-3′ and 5′-GCGGATCCGAAGCGGTTTGCATTCTCCCTTC-CTGC-3′.

TgMIC8 5′-GGTTGACCTTTTCGCCGC-3′ and 5′-GGCACC-TGCCGCACACA-3′.

TgMIC9 5′-CATGAACGCATGCGCAGCC-3′ and 5′-GCAGT-GGACGCGTGAGAAGG-3′.

Construction of plasmids

Fragments corresponding to partial coding sequences of the MICs were amplified by PCR using RH cDNA as template, with specific primers containing restriction sites. The PCR products were digested with restriction enzymes and ligated into pGEX4T-1 (AMRAD Pharmacia Biotech, Melbourne, Victoria, Australia) or pET in which a C-terminal his-tag was included (Invitrogen, Carlsbad, CA) vectors. MIC6 EGF domains: MIC6-1 5′-CGGGATCCCATGCATGAATCG-TTACTGTGGCTGAAG-3′; MIC6-2 5′-CGGGATCCATGCATGT-CCACTTCCTTCCTCT-3′.

MIC6 cytoplasmic tail: MIC6-3 5′-CGGAATTCGTTGCATA-CATGAGAAAGAGTGGGAGC-3′; MIC6-4 5′-CTGCAGTCGACC-TTAATCCCATGTTTTGCTATCCAAAT-3′.

MIC7 EGF domains: MIC7-1 CGGGATCCCTCGAGGCGGA-CTTGTGCCGCAATGAT; MIC7-2 5′-CGGGATCCCCGGGACCC-AATTGACTGAGGTGTGT-3′.

MIC7 cytoplasmic tail: MIC7-3 5′-GGCGGATCCGGAGGAAT-TTCTTACGCCAGAAACA-3′ MIC7-4 5′-CGCGGATCCTTAGGA-CCAGATACCGCCCGA-3′.

MIC8 EGF domains: MIC8-1 5′-CGGGATCCTGCAGAGCACC-ACAGCCAAAGGGG-3′. MIC8-2 5′-GGCGGATCCAAGGATATC-AACGAATGTGAAGAACC-3′.

MIC9 EGF domains: MIC9-1 5′-CGGAATTCTGTTCCTCTC-AACCGTGTGGT-3′;

MIC9-2 5′-AAAACTGCAGACTCGAGCCACTGGGTCATGTC-TGACTCG-3′.

Expression vectors for *T. gondii* were constructed based on the pTHXGPRT vector previously described (Hettmann et al., 2000). The coding sequences for the MICs were cloned between *Eco*RI site and *Pac*I sites. The construct pTMIC6Ty-1 contains a Ty-1 epitope tag,

which was inserted between the EGF-like domain 3 and the acidic region as previously described (Reiss et al., 2001). The pTmycMIC6 was obtained by cloning the cDNA of MIC6 downstream of the myc tag of the pT expression vector, as described before (Soldati et al., 1998). The two *T. gondii* expression constructs pTMIC8-Ty1 and pTMIC9-Ty-1 were cloned into pTGFPHX vector previously described (Hettmann et al., 2000). The coding sequence of MIC9 was amplified from a cosmid clone and the cDNA of MIC7 were amplified by RT-PCR using primers hybridizing upstream of the signal peptide and at the STOP codon of each respective genes. The following primers were used: MIC9-3 5′-CGGAATTCCCTTTTTCGACAAA-ATGAGGGTTTCGTTTAACGGACC-3′ and MIC9-4 5′-CCTT-AATTAAGCTGCAGGATACATTCCTTCAAAATCGTGTGCAT-3′ MIC7-3 5′-CGGCAATTGCCTTTTTCGACAAAATGGGAGGCT-GGGAGTCAAAAG-3′ and MIC7-4 5′-CCTTAATTAAGATGCA-TTCCCTCTGAACGAATGCGCCG-3′. A Ty-1 epitope was introduced at the C-terminus just preceding the stop codon. pTMIC8TyGPI was obtained in two steps. An inverse PCR reaction on pTMIC8 allowed introducing a Ty1 tag and a unique *Nsi*I site. MIC8-5 5′-CCTTAATTAAGATGCATCGAGGGGGTCCT-GGTTGGTGTGGACTTCTGGTAAACACTGCAACTTTGACCC-3′, MIC8-6 5′CCAATGCATCTACTGATCAGACAGATTTCGATTCC-3′. Subsequently, the transmembrane and cytosolic domains (TMCD) of MIC8 were exchanged with the GPI anchor signal of SAG1 using the *Nsi*I and *Bam*HI sites. The construct pTMIC6ΔE1-2GPI was constructed by inverse PCR using pTMIC6GPI as template, as previously described (Reiss et al., 2001).

Transfection and selection of recombinant parasites

T. gondii tachyzoites (RH*hxgprt*⁻) were transfected by electroporation as previously described (Soldati and Boothroyd, 1993). Hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) was used as positive selectable marker gene in the presence of mycophenolic acid and xanthine as described (Donald et al., 1996). Freshly egressed parasites ($5{\times}10^7$) of RHhxgprt⁻ strain were resuspended in cytomix buffer in presence of 80 µg of plasmid carrying the HXGPRT selectable marker gene and the expression cassette. Alternatively, in *HXGPRT*⁺ background strains, chloramphenicol acetyltransferase was used as selectable marker gene in co-transformation.

Expression and purification of recombinant fusion proteins in *E. coli*

Expression of fusion proteins was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The gluthathione S-transferase (GST) fusion protein were purified by affinity chromatography on glutathione agarose according to the manufacturer (Stratagene). The hexahistidine fusion was purified with NiTA agarose column, under denaturing conditions according to the manufacturer's instructions. The purity and integrity of fusion proteins were assessed by Coomassie blue staining of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was determined using a BioRad protein assay.

Generation of polyclonal antisera

For production of antisera to the MIC fragments, rabbits were injected with 0.1-1 mg of recombinant proteins in complete Freund's adjuvant and boosted up to five times. The reactivity of antibodies was tested by immunoblots and IFA. Antibodies raised against the EGF-like domains of the MICs were named α-Nter, and the antibodies raised against the cytoplasmic tails were named α-Cter.

SDS-PAGE and western blotting

Protein preparations were solubilized either directly in SDS loading buffer or in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% NP-40, 0.5% sodium deoxycholate, protease inhibitors Complete, Roche) followed by centrifugation for 15 minutes at 13,000 *g* at 4°C. The supernatant was mixed with SDS-PAGE loading buffer in presence of DTT. Western blot analysis was undertaken as previously described (Reiss et al., 2001).

Determination of transmembrane proteins topology

Transient permeabilization followed by proteinase K treatments were carried out as previously described for *P. falciparum* (Gunther et al., 1991). Freshly lysed parasites were washed and resuspended in PBS at 10^8/ml and slowly frozen at –80°C. The frozen cells were thawed on ice and incubated for 60 minutes in presence of increasing concentrations of proteinase K (0.05 to 1 mg/ml) in the presence or absence of a final concentration of 0.3% Triton X-100. The total cell lysate was precipitated with trichloroacetic acid (TCA) and analyzed by western blot.

Indirect-immunofluorescence assays

For the indirect immunofluorescence assay, tachyzoites were used to infect human foreskin fibroblast (HFF) cells that were growing on cover slides in 24-well plates. After 24-36 hours, cells were fixed with 4% paraformaldehyde or 4% paraformaldehyde, 0.005% glutaraldehyde in PBS for 15 minutes, permeabilized for 20 minutes with PBS containing 0.2% Triton X-100 and blocked in PBS containing 0.2% Triton and 2% Albumin fraction V. The antibodies were diluted in the permeabilization buffer with BSA. The primary antibodies were polyclonal rabbit α-*T. gondii* MICs (dilution 1:500 to 1:1000) and mouse monoclonal α-MIC2 (dilution 1:3000). The secondary antibodies were Alexa™ 488 goat α-mouse IgG and Alexa™ 594 goat α-rabbit IgG antibodies (Molecular Probes, Netherlands) diluted 1:1000. Cells were washed three times with PBS+0.2% Triton X-100 and mounted in a mounting solution (fluoromount G, Southern Biotechnologies). Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM/IRB) using a 63× Plan-Apo objective with NA 1.40. Optical sections were recorded at 250 nm per vertical step with four 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 processing of images.

Secretion assays

For large-scale preparation of excretory-secretory antigens (ESA), approximately $5{\times}10^9$ tachyzoites were washed and resuspended in 1 ml of HHE and stimulated to discharge micronemes by addition of ethanol to a final concentration of 1.0% and incubation at 37°C for 30 minutes as described previously (Carruthers et al., 1999). Cells were removed by centrifugation at 2000 *g* and the supernatant was kept frozen at –70°C.

Immunoaffinity purification of microneme protein

Immunoaffinity purification of MIC6Ty was obtained by using α-Ty1 monoclonal antibodies BB2, crosslinked to protein A sepharose (Pharmacia) with dimethylpimelimidate as described in the laboratory manual (Harlow and Lane, 1988). Freshly lysed parasites ($9{\times}10^9$) from mic6ko strain expressing MIC6Ty were harvested, washed once in PBS and lysed in RIPA for 30 minutes on ice. The lysate was then spun at 50,000 *g* for 60 minutes at 4°C and the supernatant was incubated with antibodies coupled to the beads for 12 hours at 4°C with gentle shaking. The beads were washed three times with 15 ml of RIPA. Proteins were eluted in by boiling for 5 minutes in non-reducing SDS-PAGE sample buffer. Samples were separated on 12% SDS-PAGE gels and stained with Coomassie R-250.

Mass spectrometry analysis

Mass spectrometry was carried out at the ZMBH Biopolymer facility, similar to described procedures (Shevchenko et al., 1996; Wilm et al., 1996). Briefly, the bands were cut out of a stained SDS-PAGE gel. The gel piece was washed twice with H_2O, twice with 50% acetonitrile and once with 100% acetonitrile. Proteins were reduced in gel with 10 mM DTT in 100 mM NH_4HCO_3 at 56°C for 1 hour. Alkylation was performed in 10 mM iodoacetamide. The gel piece was washed again as described above. In gel, tryptic digest was performed with 1.25 ng/μl trypsin in 50 mM ammonium bicarbonate overnight at 37°C and stopped by addition of acetic acid. The digestion mixture was desalted on a capillary packed with POROS R2 sorbent (Applied Biosystems). Peptides were eluted with 70% methanol/1% acetic acid. Mass spectrometric analysis was performed according to the manufacturer, on a Q-STAR (Applied Biosystems) mass spectrometer equipped with a Nano ESI ion source.

Results

Identification of four genes coding for putative transmembrane proteins and containing EGF-like domains in *T. gondii*

To expand the repertoire of micronemal proteins potentially involved in *T. gondii* gliding motility and invasion, we searched the Toxoplasma ESTs database for proteins containing TRAP-family-like transmembrane and cytoplasmic sequences. The first of these novel micronemal proteins was encoded in a contig of four ESTs (Ctoxqual2_2870). Using a

probe based on this sequence, we screened a cDNA library and isolated and sequenced two full-length cDNAs. The deduced amino acid sequence predicts a protein of 34 kDa with an N-terminal predicted endoplasmic reticulum targeting signal and a putative transmembrane spanning domain near the C-terminus. Based on its localization in the micronemes, and the status of the nomenclature, we named the new *T. gondii* protein, TgMIC6 (Reiss et al., 2001). The extracellular region of this protein is composed of three EGF-like domains and one acidic region, preceding the membrane-spanning domain. A number of apicomplexan proteins containing EGF-like domains have been previously reported (Fig. 1A). In *Plasmodium*, these proteins are anchored to the surface of ookinetes or merozoites by a glycosylphosphatidylinositol (GPI) lipid. A larger protein containing 10 EGF-like domains is also present in the *P. faciparum* genome (Pf125). The deduced amino acid sequence predicted a 125 kDa protein exhibiting a transmembrane domain and a very short tyrosine-rich cytoplasmic tail; however, these domains lack the conserved residues typically found in some other microneme proteins. EtMIC4 is a 200 kDa protein containing 31 EGF-like and 12 thrombospondin-like domains that was recently identified in the micronemes of *E. tenella* (Tomley et al., 2001). The cytoplasmic tail of TgMIC6 contains two recently mapped signals that are essential and sufficient for sorting to the micronemes (Di Cristina et al., 2000). The protein also

Fig. 1. Comparison of EGF-like proteins from apicomplexan parasites. (A) Apicomplexan proteins containing EGF-like domains. *P. falciparum* merozoite surface antigen PfMSP1, *P. falciparum* circumsporozoite protein PfCSP, *Plasmodium* ookinete surface antigens PfP21/P25, *P. falciparum* 125 kDa protein, Pf125 (PFC1045C, ACO97302), *E. tenella* microneme protein EtMIC4, and *T. gondii* microneme proteins TgMIC3, TgMIC6, TgMIC7, TgMIC8 and TgMIC9. (B) Amino acid sequences alignment of the cytoplasmic tails of transmembrane microneme proteins across the Apicomplexa. The amino acids colored in green correspond to the membrane-spanning domain and terminate with an almost strictly conserved tyrosine residue in blue. The conserved tryptophan residue is colored in red. (C) Alignment of the lectin-like domains present on TgMIC3 (TGO132530), TgMIC8 (AAK19757), NcMIC3 and *E. tenella* ESTs.
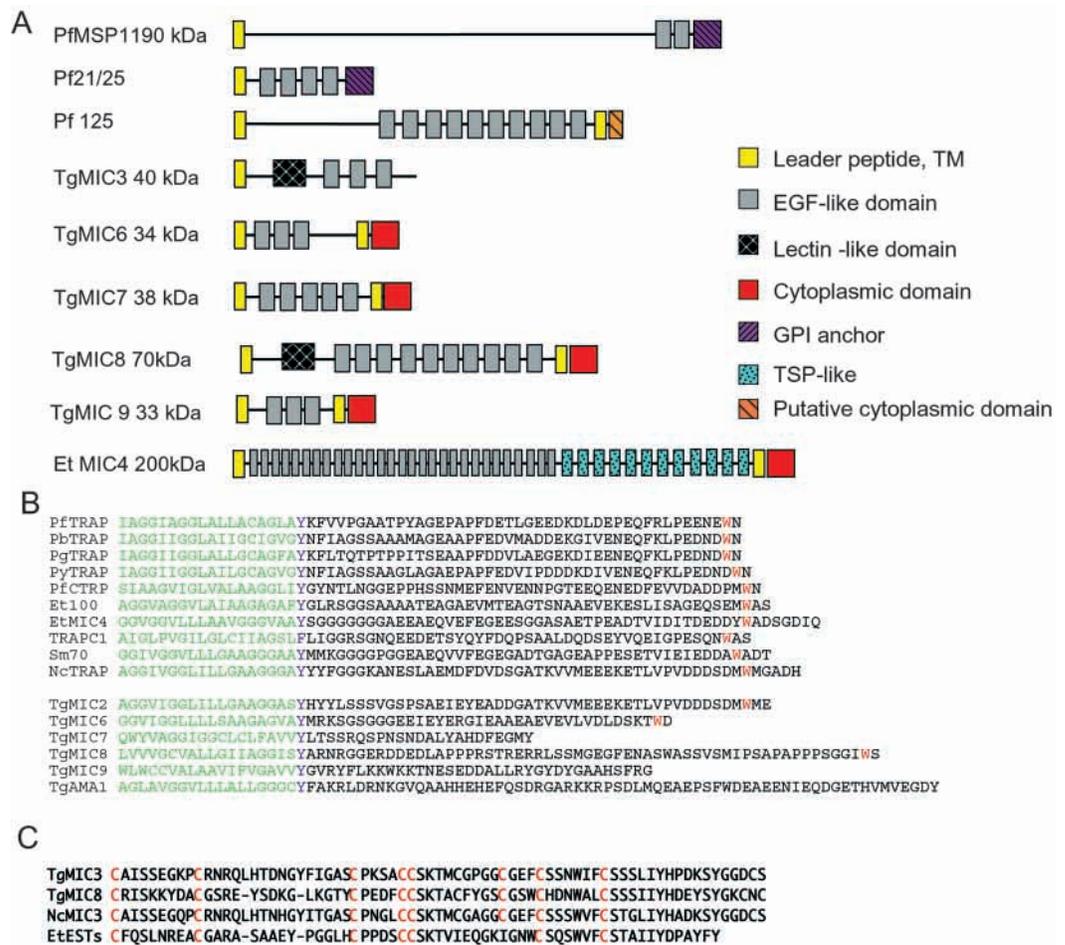
contains a strictly conserved tryptophan residue, which has been shown to play an important role in the function of PbTRAP protein in gliding motility and host cell invasion by *Plasmodium berghei* sporozoites (Kappe et al., 1999) (Fig. 1B). We have recently disrupted *TgMIC6* gene by double crossover homologous recombination and showed that this protein plays a crucial role as an escorter of the two soluble adhesins, TgMIC1 and TgMIC4 (Reiss et al., 2001).

Further searching of the EST database for EGF-like domains led to the identification of two additional putative transmembrane proteins structurally similar to TgMIC6. These new proteins were named TgMIC7 and TgMIC8. The sequences obtained from the corresponding ESTs were used to screen a genomic library and several clones for each gene were isolated and sequenced. While sequencing the *TgMIC8* locus, a fourth gene coding for a putative transmembrane protein exhibiting three EGF-like domains (TgMIC9) was identified upstream of the *TgMIC8* gene. The cDNAs corresponding to TgMIC7, TgMIC8 and TgMIC9 were obtained by RT-PCR, cloned and sequenced. *TgMIC7* is the only one of the four genes that contains introns. The predicted amino acid sequence of each of the four genes and their signal peptide and putative transmembrane spanning domains are depicted in Fig. 2. The amino acid sequences of their transmembrane and cytoplasmic domains are aligned and compared with the same domains present in other apicomplexan microneme proteins (Fig. 1B). In addition to the EGF-like domains, TgMIC8 also possesses a lectin-like domain similar to the one recently described in the non-membrane protein TgMIC3 (Garcia-Reguet et al., 2000). The cysteine rich lectin-like domain of TgMIC3 has recently been implicated in binding to host cells (Soldati et al., 2001). This lectin domain is also present on a microneme protein of *Neospora caninum* (NcMIC3), which is closely related to TgMIC3 (Sonda et al., 2000), and in an as yet uncharacterized *E. tenella* partial open reading frame present in the EST database (Fig. 1C).

Members of this microneme protein family display different patterns of expression in the proliferative and encysted life stages of *T. gondii*

Two life stages of *T. gondii* are present in intermediate hosts. The tachyzoite, a rapidly dividing form is responsible for acute infection and the slowly growing encysted bradyzoite form is associated with chronic infection. We examined the stage-specific expression of the members of this novel gene family by measuring the level of their transcripts in the two life stages by semi-quantitative RT-PCR. 76K strain bradyzoite cysts were isolated from mice chronically infected for two months and 76K tachyzoites were obtained from in vitro culture in HFF cells. To ensure that equal quantities of each mRNAs were being compared, the housekeeping gene α-tubulin was used as control (Fig. 3). The results revealed that TgMIC6 is preferentially expressed in tachyzoites, whereas TgMIC8 transcripts are evenly distributed between the two stages. By contrast, TgMIC7 and TgMIC9 are preferentially or exclusively transcribed in the encysted form of *T. gondii*. TgMIC2 and TgMIC4 were also included as controls because they are expressed in both parasite stages (Brecht et al., 2001).

TgMIC6

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1    MRLFRCCAAAVVAAESLLWLKNGSPFFAFLPGNGEIADNCSGNPCGGTAA    50
51   GTCINTPSGYDCRCEPGYVLGVENDQVTCMMPSGVPMANFVQLSETPAAC   100
101  SSNPCGPEAAGTCKETNSGYICRCNQGYRISLDGTGNVTCIVRQESGCEE   150
151  NGCGPPDAVQSCRRLTGTAGRLCVCKENFIATIDASAHITCKRVPPHYRK   200
251  PPFEFGKGGHPVDSEPSKRQREDEGESREPESDSTEPGRDQERRTPLEES   250
301  QEPEGSTPDSQQSRGGSGSDSTESEEQGKEREEGSGHAGAIAGGVIGGLL   300
401  LLSAAGAGVAYMRKSGSGGGEEIEYERGIEAAEASEVEVLVDLDSKTWD    349
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TgMIC7

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1    MGGWESKDACESGGTAFCGTVANSAGCIALSKEFYCTCLPGFQPEGRRMN    50
51   SSCVDIDECARGIGLCFSKIENSECVNTPGSFECRCNEYRTLKNNVCEDI   100
101  NECLDNRGGCAEHTDCINNDGAAVTCRCLPGYTGNDGKPGRACKDINECE   150
151  EPDHCPENSVCVNTPGSYRCECKTGFAKDAEGMCTGKDFCVTGENGCDPN   200
201  FATCTPVVGSYACACKDGFQGSGRKGECKPLPGSEYKACELLGLHCGRYQ   250
251  GCASDNAGSYRCAALTKVQQVKVLLSEGPGTETPLWLWCCVALAAVIFVG   300
301  AVVYGVRYFLKKWKKTNESEDDALLRYGYDYGAAHSFRG    339
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TgMIC8

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1    MKANRIWCFFAWRMVVRASFLKEMDSIFVSAIRQNVQHTHSALLAKLKEP    50
51   PDPDDENSWLCRISKKYDACGSREYSDKGLKGTYCPEDFCCSKTACFYGS   150
101  CGSWCHDNWALCSSSIIYHDEYSYGKCNCKRFQENCDVNAICVHANREDG   200
151  GAYCQCKEGYWGDGKSCKIDFCQLQPCGAGTCTRTDEGYKCDCPETHKLI   250
201  VVEDKETCKAKPDFCAEEPCGPPSMVENCVNTDDSYECVCKQGYEVRNGR   300
251  CEEIDLCADKPCGPDEGVHECVTERQPKLRYRCTCKAGFDLTTLPDGVSQ   350
301  KCLKNFCYEEPCGTRDLVESCKSKAYGYSCLCAAGAMVQVINGKEKCIKA   400
351  DLCRNDPCGPETAVIQCYSHGTSYRCLCKAGYTEVFVNGKSSCQKGDPCT   450
401  LNMCGGNEAVQECTTDGTAYGCTCKPGYSIAIKHGQKFCNPEEECASHCG   450
451  SAAAVKSCEILDSGGYQCTCNPGYVMRYSDYVKGCVEGNQCSLNPCGEQE   500
501  AVQRCIPEGDTYDCECNPGFVKRVLPDGNFICADPASCVGNPCGSSDAVD   550
551  ACIAGTSTYTCRCKDGYTPQSIGSKLQCLPESTDQTDFDSKHKPEDNKGR   600
601  YSKGTIALVVVGCVALLGIIAGGISYARNRGGERDDEDLAPPPRSTRERR   650
651  LSSMGEGFENASWASSVSMIPSAPAPPPSGGIWS    684
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TgMIC9

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1    MRVSFNGPVHRTGFVLVCVIGSLCWCIAPAPECVFPVTGLGPAAWNHPGS    50
51   LGQMRAESKGVLSGTGICDRNPCGPHGNCIPNLSTDTYTWSCEGGYVEAA   100
101  VSGKPTCQRKDPCDTSPCGTKEAVKECRPNGQYYACICQAGYQVVSTDTG   150
151  SRCQRIKSTTTPCSSQPCGGPEAVEACLVTGPTTYSCTCASGYETITTEQ   200
201  GERCEKTDPCLSNPCGASKYVQSCTATNTGYTCECVKGAKKTGSDSAPMC   250
251  VAGESDMTQWYVAGGIGGCLCLFAVVYLTSSRQSPNSNDALYAHDFEGMY   300
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Fig. 2. Nucleotide and predicted amino acid sequences of TgMIC6, TgMIC7, TgMIC8 and TgMIC9. The predicted signal peptides and transmembrane spanning domains are underlined. The accession numbers are: TgMIC6, AAD28185; TgMIC7, AF357911; TgMIC8, AAK19757; and TgMIC9, AAK19758.

TgMIC6 and TgMIC8 undergo proteolytic processing at their C-terminus upon secretion

To characterize and study the properties of TgMIC6 and TgMIC8, we expressed and purified bacterial recombinant fragments of the extracellular or cytoplasmic domains of these
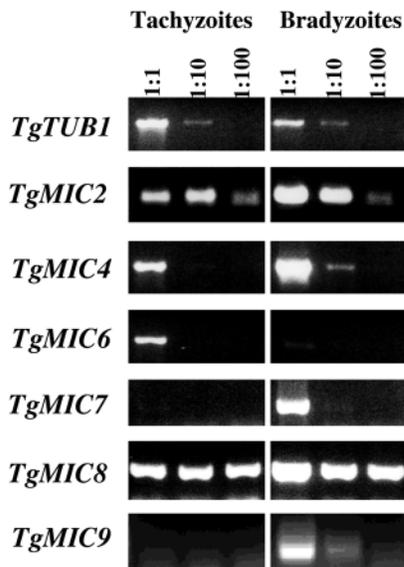
Fig. 3. Developmental regulation of the transcripts coding for EGF-like domains containing proteins. Semi-quantitative RT-PCR analysis of transcripts corresponding to *TgMIC2*, *TgMIC4*, *TgMIC6*, *TgMIC7*, *TgMIC8* and *TgMIC9* genes, performed with tenfold serial dilutions of in vitro tachyzoite and in vivo bradyzoite cDNAs. To ensure that equal amounts of cDNA from each parasitic stage were being compared, the housekeeping α-tubulin primers were used as controls.

two proteins. The cytoplasmic tails of these proteins were fused to GST and purified under native conditions, while the entire extracellular region of TgMIC6 or five EGF-like domains of TgMIC8 were fused to a hexahistidine tag and purified under denaturing conditions. These fragments were used to raise specific rabbit antisera to characterize the expression of the corresponding proteins by western blotting and indirect immunofluorescence. All antisera reacted similarly on lysates prepared from RH and Prugniaud strains of *T. gondii* and gave no signal on lysates prepared from Vero cells. Western blot analysis with α-NterMIC6 revealed one predominant product of 45 kDa, a faint band at 53 kDa, and two crossreacting bands of higher molecular weight, which persist in the mic6ko lysate. The α-CterMIC6 antibodies recognized both the 53 and the 45 kDa products (Fig. 4A). Polyclonal antisera raised against either the cytoplasmic tail or the EGF-like domains of TgMIC8 detected a single product of about 70 kDa (Fig. 4B).

To determine the compartments where these processing events take place, we stimulated microneme secretion and compared the contents of micronemes with the material released in the supernatant (ESA) after stimulation with ethanol. As previously shown for TgMIC2, TgMIC6 and TgMIC8 lost their C-terminal domain upon release from the parasite surface since a soluble, secreted 35 kDa product of TgMIC6 was not detectable with the α-CterMIC6 antibodies and a ~65 kDa secreted processed form of TgMIC8 was not detectable with α-CterMIC8 antibodies (Fig. 4C,D). The three distinct forms of TgMIC6 corresponding to ~53-, 45- and 35-kDa are absent in a *T. gondii* mic6ko mutant strain, confirming that these polypeptides are the products of the same gene (Reiss et al., 2001). The sera raised against the cytoplasmic tail

of TgMIC6 and TgMIC8 failed to recognize the 35 and 65 kDa forms, respectively, indicating that a cleavage occurred close to the putative transmembrane spanning domain, in a way similar to the processing of TgMIC2 (Carruthers et al., 2000).

The protease responsible for TgMIC6 and TgMIC8 C-terminal cleavage is presumably shared between all transmembrane microneme proteins and corresponds to the MPP1 activity. MPP1 was recently defined as the proteolytic activity responsible for the removal of the C-terminus of TgMIC2 (Carruthers et al., 2000). While TgMIC6 was previously demonstrated to localize in the micronemes (Reiss et al., 2001), indirect double immunofluorescence analysis by confocal microscopy confirmed the perfect co-localization of TgMIC8 with the previously characterized microneme marker TgMIC2 (Fig. 4E).

TgMIC6 is cleaved at the N-terminus during its transport to the micronemes

In addition to the C-terminal cleavage, TgMIC6 is cleaved in parasites at the N-terminus, causing the removal of the first EGF-like domain (Reiss et al., 2001). We engineered recombinant parasites expressing TgMIC6 with a myc-tag epitope introduced immediately downstream of the signal peptide cleavage site of the protein in order to follow the biogenesis of the protein within the cells. This construct was stably transformed in mic6ko strain. We failed to detect the 53 kDa precursor by western blot with α-myc; however, a specific signal was detectable by IFA in less than 30% of the vacuoles (Fig. 5A). Interestingly, the α-myc antibodies failed to stain the micronemes, suggesting that the N-terminal processing occurred during transport along the secretory pathway, most likely in the trans-Golgi network (TGN). A series of overlays have been chosen to illustrate several points. In Fig. 5B, the transgenic product mycMIC6 (green) is exclusively targeted to the micronemes as seen by colocalization (yellow) with TgMIC2 (red). Additionally, mycMIC6 is also present in significant amounts in the ER and Golgi. Fig. 5C provides evidence that the precursor of mycMIC6, as detected by α-myc (red), accumulates in the early compartments of the secretory pathway but was absent from the micronemes (green) labeled with α-TgMIC4. Interestingly, the precursor of mycMIC6 appears to move in wave along the secretory pathway, likely in a cell cycle-dependent fashion. This potential cell cycle dependency of TgMIC6 traffic has been previously observed in mic1ko (Reiss et al., 2001). Definitive evidence for this processing taking place in the late Golgi came from the analysis of the lipid anchored MIC6GPI mutant (Reiss et al., 2001). The protein covalently linked to GPI was targeted directly to the plasma membrane without routing through the micronemes and still underwent N-terminal processing, suggesting that this event occurs prior to the branch point where microneme and surface proteins segregate. By contrast and as a control, GPI-anchored MIC6 with a deletion of the first EGF domain was not processed (Fig. 5D).

The exact cleavage site at the N-terminus of TgMIC6 was determined by mass spectrometry analysis after immunoprecipitation of MIC6Ty and purification of the 35 kDa species by SDS-PAGE. The protein was clearly identified by MALDI TOF mass spectrometry (data not shown). To determine the cleavage site we digested the purified 35 kDa

form from MIC6Ty with trypsin and analyzed the digestion products by ESI Hybrid Quadrupole TOF MS. We calculated all theoretical masses of peptides potentially generated upstream of the K114 (in the EGF-2 domain). Only one of these masses was detected in the mass spectrum as a doubly charged ion of 689.0 m/z, which was then sequenced by tandem MS (Fig. 5E). The resulting fragmentation pattern clearly showed that this peak results from the peptide ETPAACSSNPCGPEAAGTCK. Since a cleavage between S94 and E95 could not arise from tryptic activity, we concluded that the N-terminal cleavage site of the 35 kDa fragment occurs between the serine and the glutamic acid residues (VQLS*ETP).

TgMIC6 adopts a classical type I membrane topology both during transport along the secretory pathway and during its storage within the micronemes

The transmembrane proteins stored in the secretory organelles of *T. gondii* including the micronemes and dense granules are unexpectedly soluble in absence of detergent. Within the micronemes, TgMIC2 is completely soluble, whereas at least 50% of TgMIC6 is readily soluble without detergent (C. Opitz and D.S., unpublished). This observation raised concerns about the topology adopted by these proteins in the membrane. To elucidate the topology of TgMIC6 during its transport and when stored in the organelles, we applied a proteinase K treatment after a transient permeabilization of the cells (Gunther et al., 1991). The cells were frozen and thawed slowly in cold. This treatment lyses cells and permits microsomes and organelles to reseal with proteins in their original orientation. In wild-type RH parasites, the majority of TgMIC6 localizes to the micronemes and is N-terminally processed into a 45 kDa form. In mic1ko strain, the 53 kDa precursor (N-terminally unprocessed) of TgMIC6 accumulated predominantly in the early compartments of the secretory pathway (in the ER and Golgi apparatus) (Reiss et al., 2001). Finally, the 35 kDa product corresponds to the fully processed form released by the parasites (Fig. 6A). After treatment with proteinase K in absence of detergent, both the precursor (Fig. 6B, right panel) and the mature forms (Fig. 6B, left panel) were converted into slightly smaller products corresponding to truncation of their C-terminal tail. This assumption was confirmed by the inability to detect these products with the α-CterMIC6 antibodies (Fig. 6C). A similar pattern of protection was observed in RH and in mic1ko, suggesting that TgMIC6 adopts a type I membrane topology both during its transport through the ER and Golgi and upon storage into the micronemes. The same
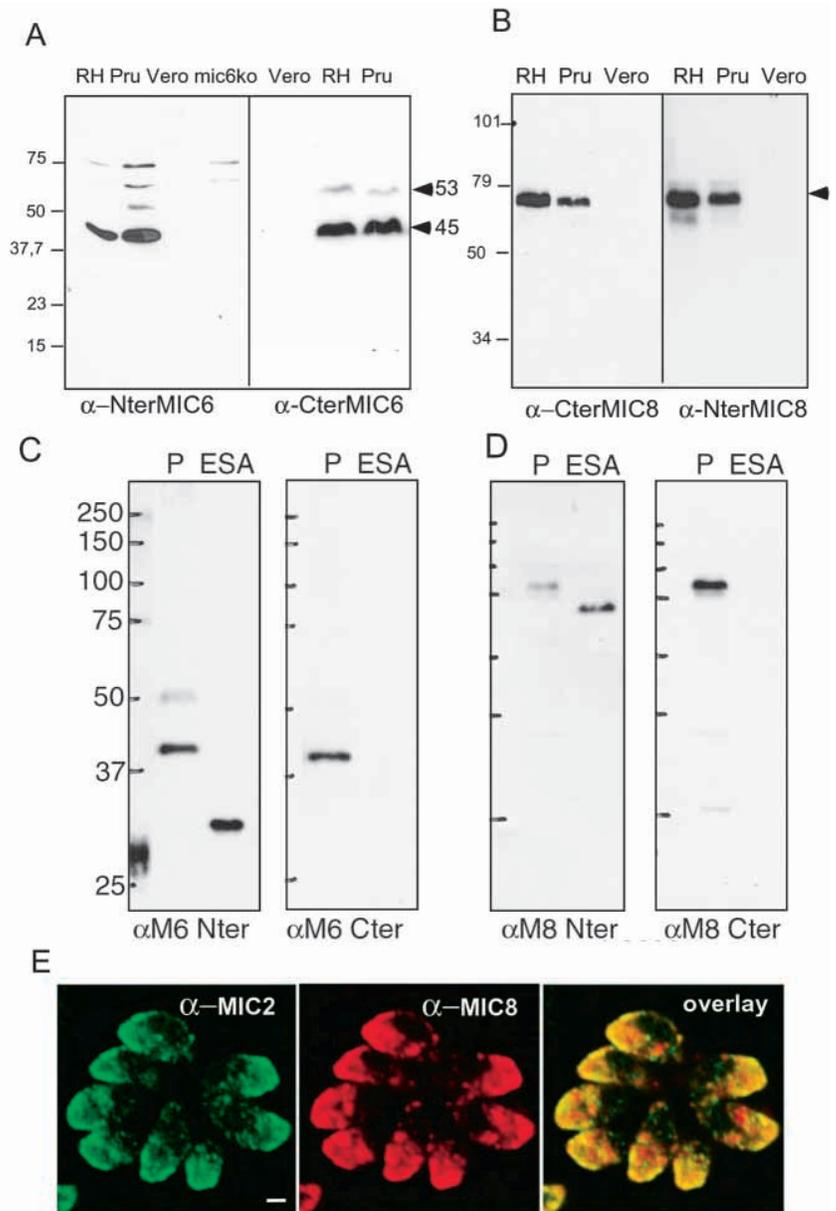
Fig. 4. TgMIC6 and TgMIC8 are microneme proteins, processed at their C-terminus upon secretion. Western blot analysis of *T. gondii* tachyzoites probed with antisera raised against NterMIC6 or the CterMIC6 (A) and with antisera raised against NterMIC8 and CterMIC8 (B). Lysates were prepared from freshly lysed parasites from RH, mic6ko in RH or Prugniaud (Pru) strains and from vero cells. (C) Western blot analysis of lysates from parasite pellets and supernatants of secretion assays (ESA). TgMIC6 is processed from a 53 kDa precursor into a 45 kDa product in the parasites during its transport to the micronemes. In the ESA, the 35 kDa form is detectable with α-N-terMIC6 but failed to be recognized by the α-CterMIC6 antibodies. (D) A similar analysis was performed on TgMIC8 and showed that the ~65 kDa secreted form of TgMIC8 is not detectable with α-CterMIC8, establishing that the C-terminal domain has been removed. (E) Immunofluorescent labeling of permeabilized HFF cells infected with tachyzoites from RH strain. Double labeling with mouse α-MIC3 and rabbit α-MIC8 confirmed the microneme localization of TgMIC8. The antibodies were visualized with Alexa™ 488 goat α-mouse IgG and Alexa™ 594 goat α-rabbit IgG antibodies. Bar, 1 μm.

experiment was repeated several times including using PMSF at the end of the reaction to neutralize the proteinase K. Other

microneme proteins were also analyzed as controls. In Fig. 6C, parasites expressing MIC6Ty in the mic1ko mutant were used to analyze both TgMIC6Ty and TgM2AP, a newly characterized microneme protein tightly associated with TgMIC2 along the secretory pathway and in the micronemes (Rabenau et al., 2001). Despite its high susceptibility to proteolysis, TgM2AP is completely protected in this assay, which is consistent with its retention in the lumenal side via binding to TgMIC2 (Fig. 6D). A classical type II transmembrane protein, Toxomepsin (X. Jäkle and D.S., unpublished), was also used as a control in this assay and it exhibited the expected results with removal of its large cytoplasmic domain (data not shown).

Epitope-tagged TgMIC7 and TgMIC9 are targeted to the micronemes

We raised polyclonal antibodies against the EGF-like domains of TgMIC7 and TgMIC9 to characterize these proteins. As expected from the RT-PCR results, both proteins are poorly or not expressed in tachyzoites of the Prugniaud (Fig. 7A) or RH (data not shown) strains. The polyclonal antibodies raised against TgMIC9 revealed a strong signal around 70 kDa. This signal is most likely due to an immuno-crossreaction with another parasite antigen since it is not detectable in Vero cells lysates (data not shown) and the anti Ty-1 antibodies failed to detect such a high molecular weight product in recombinant parasites expressing TgMIC9Ty. To analyze these proteins in tachyzoites, we generated stable parasite cell lines expressing TgMIC7Ty and TgMIC9Ty under the control of the constitutive tubulin promoter with the Ty-1 epitope tag positioned at the C-terminus of each protein. Western blot analysis of these recombinant parasite lysates revealed the presence of polypeptides of the expected size as anticipated from the predicted amino acid compositions of their coding sequences. IFA analysis of recombinant parasites expressing epitope tagged TgMIC7Ty and TgMIC9Ty confirmed their localization to the micronemes (Fig. 7B,C). TgMIC9Ty also partially accumulated to the rhoptries as seen by colocalization with ROP2 (Fig. 7D). This imperfect sorting of TgMIC9Ty might reflect some problems of overexpression, stage specificity or interference due to the presence of the tag.

TgMIC7 and TgMIC9 exhibit shorter tails than the other transmembrane microneme proteins and they lack the strictly conserved tryptophan residue at the extreme C-terminus (Fig. 1B). We did not detect the secreted forms of
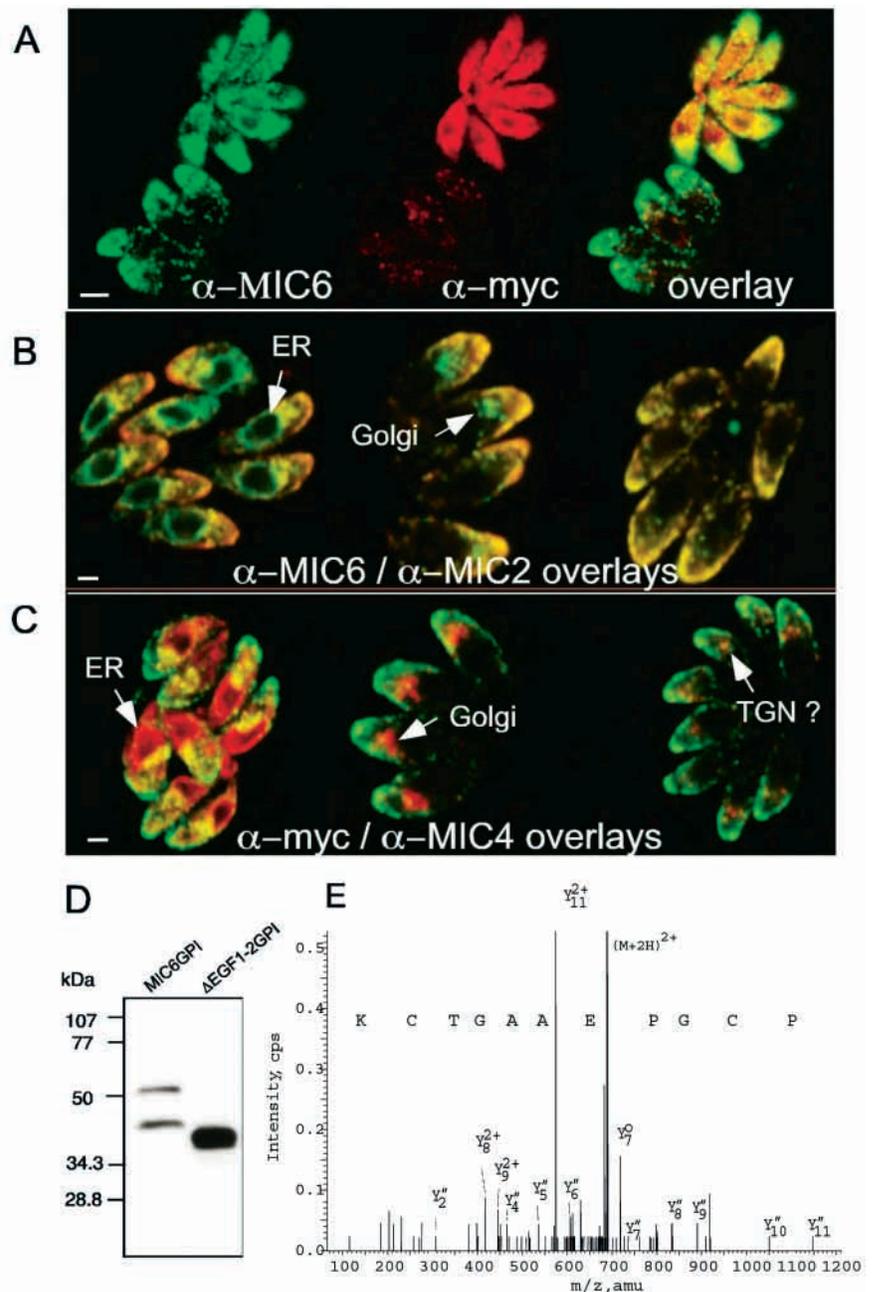
Fig. 5. TgMIC6 is N-terminally processed in the late Golgi. (A,B,C). Double IFA analyzed by confocal microscopy on intracellular parasites expressing mycMIC6. (A) The precursor of mycMIC6 (in red) is detectable only in less than 30% of the vacuoles, whereas all parasites are positive for MIC6 (green). (B) Overlays using α-MIC6 (green) and α-MIC2 (red). (C) Overlays using α-myc (red) and α-MIC4 (green). The compartments of the secretory pathway are indicated with arrows. (D) Western blot analysis of lysates from stable transgenic mic6ko parasites transformed with pTMIC6GPI or pTMIC6ΔEGF1-2GPI (MIC6 lacking the first two EGF-like domains and the TMCD domains replaced by a GPI anchoring signal from SAG1). MIC6GPI was targeted to the plasma membrane but nevertheless underwent N-terminal processing. By contrast, the deletion of EGF-1-2 removes the processing site, and no processed form was detectable. (E) Mass spectrometry fragmentation peaks of the MIC6 specific peptide corresponding to the N-terminal cleavage site of the protein.

these proteins, which might reflect a defect in secretion or in processing due to expression in the inappropriate stage of differentiation. Currently, the analysis of the endogenous

Fig. 6. Determination of type I membrane topology of TgMIC6, both during its transport and during its storage in the micronemes. (A) The three forms of TgMIC6. The 53, 45 and 35 kDa forms are present in the ER/Golgi, in micronemes and secreted, respectively. The two processing sites are indicated by arrows. (B) Western blot analysis of lysate from wild-type parasites (left panel) after transient permeabilization and proteinase K (0.1 mg/ml and 0.05 mg/ml) treatment either in the presence or absence of detergent (0.2% Triton X-100). The same analysis was repeated using mic1ko mutant parasites (right panel) in which TgMIC6 was retained in the early compartments of the secretory pathway and consequently was not processed at the N-terminus. The western blots were probed with α-NterMIC6 antibodies. The same material was analyzed in the lower panels with the α-CterMIC6 antibodies, establishing that the tail of TgMIC6 was degraded by proteinase K treatment both in RH and mic1ko strains. (C) A similar experiment was repeated on the recombinant cell line expressing MIC6Ty in mic1ko. In this experiment, TgM2AP, a 43 kDa m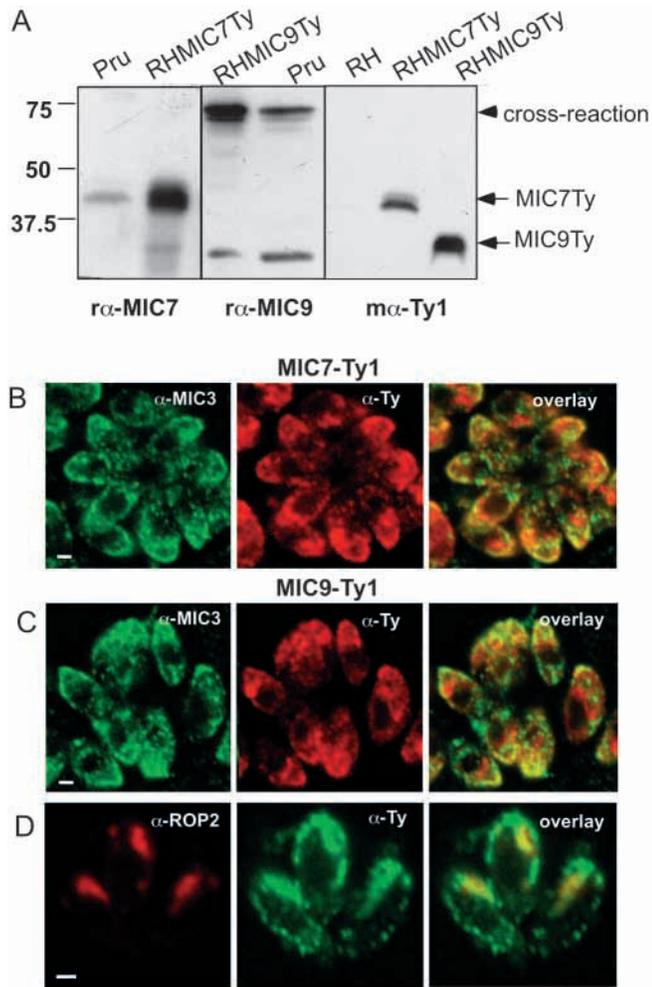icroneme protein associated with the lumenal domain of TgMIC2, was included as control to demonstrate the integrity of the micronemes.

TgMIC7 and TgMIC9 in bradyzoites is hampered by the extremely limited source of material available for this stage. In addition, polyclonal antibodies to TgMIC7 and TgMIC9 did not deliver signals on bradyzoite cysts by immunoelectron microscopy.

TgMIC8 serves as an escorter for the adhesin MIC3

TgMIC6 plays an essential escorter role in the targeting of TgMIC1 and TgMIC4 to the micronemes (Reiss et al., 2001). The overall structural homology between TgMIC8 and TgMIC6 prompted us to investigate whether MIC8 would fulfill a similar function. To rapidly address this question, we engineered a vector to express TgMIC8 covalently linked to a lipid by fusing the MIC8ΔTMCD (deleted transmembrane and cytoplasmic domains) to SAG1 glycosylphoshpatidylinositol (GPI) anchor signal. As anticipated, the expression of MIC8GPI resulted in the plasma membrane localization of the transgenic product (Fig. 8A). We then screened for any of the known microneme proteins that would redistribute to the plasma membrane as a consequence of the illegitimate presence of TgMIC8. TgMIC1, TgMIC2, TgMIC4, TgMIC5 and TgMIC6 are correctly sorted to the micronemes in the presence of MIC8GPI (data not shown). So far, TgMIC3 is the only microneme protein we have observed rerouted to the plasma membrane in this transformant. By contrast, TgMIC3 was typically localized to the micronemes in wild-type parasites (Fig. 8A). Most interestingly, like TgMIC1 and TgMIC4, TgMIC3 is a protein that lacks a membrane-spanning

domain and is capable of binding host cells (Garcia-Reguet et al., 2000). The extensive relocalization of TgMIC3 to the plasma membrane in the presence of MIC8GPI strongly suggests the existence of a stable complex between the two proteins. In contrast to MIC6GPI, we failed to generate stable cell lines expressing MIC8GPI, possibly because of the constitutive presence of the MIC3-MIC8 complex at the surface of the parasite is not tolerated. By reducing the amount of vector used in the transfection and waiting 48 hours post-electroporation, we noticed that the pattern of MIC8GPI localization was significantly different in the parasites expressing lower amounts of the transgene. Interestingly, MIC8GPI did not distribute evenly at the surface of the parasites but was clearly confined in regions of cell-cell contacts between parasites residing within a vacuole (Fig. 8B).

Discussion

A common feature of proteins that include EGF-like motifs is their involvement in extracellular function such as adhesive and ligand-receptor interactions (Campbell and Bork, 1993). The presence of EGF-like domains in protozoan parasite proteins was originally described on the *Plasmodium* ookinetes surface (PfP25) (Kaslow et al., 1988). Such domains have also been found in the merozoite surface protein-1 (MSP-1) of *Plasmodium* species, a lipid anchored surface antigen representing one of the leading candidates for a vaccine targeted at the erythrocytic stages of plasmodial parasite development (Blackman et al., 1991). More recently, additional

Fig. 7. TgMIC7 and TgMIC9 are microneme proteins poorly expressed in tachyzoites. (A) Western blot analysis of lysates from wild-type RH or Prugniaud strain and transgenic RH parasites stably transformed with MIC7Ty or MIC9Ty under the control of the constitutive promoter *TUB1*. (B) Double IFA by confocal microscopy of transgenic parasites expressing MIC7Ty. MIC7Ty colocalized (α-Ty, red) with the microneme marker TgMIC3 (green). (C) Double IFA of transgenic parasites expressing MIC9Ty. MIC9Ty (red) colocalized with TgMIC3 (green). (D) In addition to the microneme localization, MIC9Ty (green) partially localized to the rhoptries, as documented by double IFA with α-ROP2 (red). Bar, 1 µm.

Fig. 8. MIC8 serves as escorter for the non-membrane adhesin TgMIC3. (A) Double IFA analysis by confocal microscopy of parasites transiently transfected with pTMIC8GPI. TgMIC8 covalently linked to a GPI anchor localized perfectly at the plasma membrane of the parasites. TgMIC3 redistributed to the plasma membrane in the transiently transfected parasites, while the protein is perfectly sorted to the micronemes in a vacuole containing non-transfected parasites. (B) Three examples of vacuoles containing parasites transfected with 30 µg of pTMIC8GPI vectors and analyzed 48 hours post-electroporation. MIC8GPI is stained with the α-Ty1 (red) and accumulates at the plasma membrane specifically in the zones of contacts between parasites. The α-MIC6 is used as microneme marker (green). Bar, 1 µm.

EGF-containing surface antigens such as MSP4 and MSP5 have been characterized in *Plasmodium* species (Marshall et al., 1998). In *T. gondii*, the adhesin TgMIC3 was recently characterized as a microneme protein carrying five overlapping EGF-like domains (Garcia-Reguet et al., 2000). A transmembrane microneme protein (EtMIC4), carrying a very large extracellular domain including 31 EGF-like domains and 12 thrompospondin-like modules, has been described in *E. tenella* (Tomley et al., 2001). The report here of four novel proteins containing EGF-like domains in *T. gondii* illustrates the extensive use of these modules by apicomplexan parasites. In addition to EGF-like motifs, TgMIC8 contains a lectin-like domain also present in TgMIC3. Furthermore, several overlapping *E. tenella* ESTs are predicted to encode a protein carrying a lectin-like domain, an EGF-like motif and a stretch
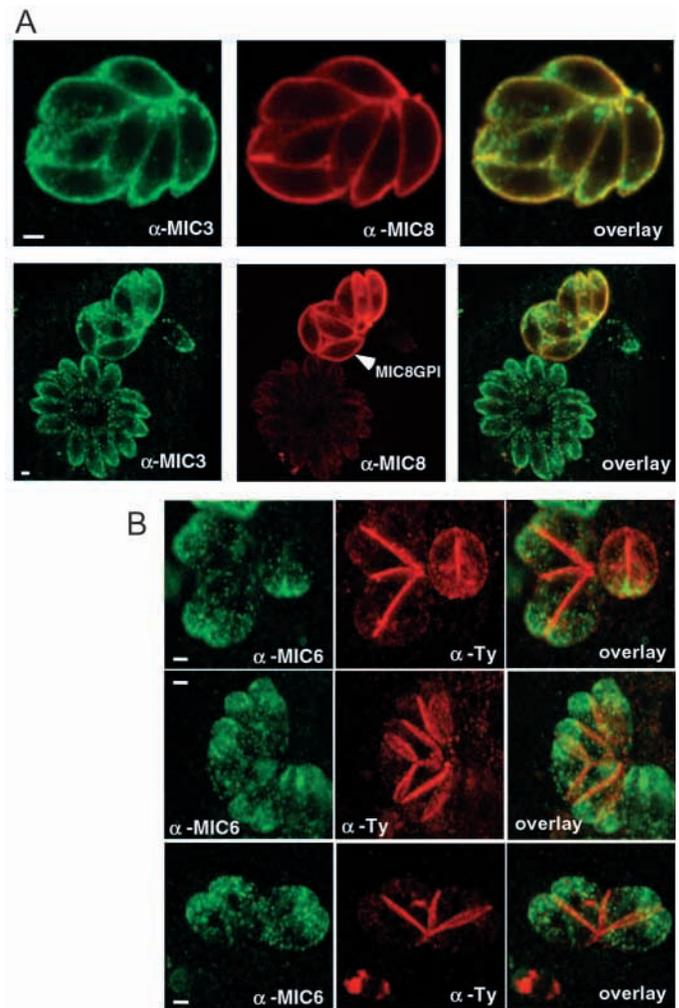
of tyrosine residues. It is also interesting to note that stretches of tyrosine residues have been previously identified in a microneme protein of *Cryptosporidum parvum*, GP900 (Barnes et al., 1998).

We recently elucidated one aspect of TgMIC6 function by gene disruption. The absence of TgMIC6 does not interfere with the survival of *T. gondii* tachyzoites in culture despite the fact that its absence caused the complete mistargeting of two adhesins to the parasitophorous vacuole. A role as escorter implies that the protein carries sorting signals capable of recruiting the cytoplasmic components of the sorting machinery. These signals have been mapped to the C-terminal

domain of TgMIC2 and are conserved on TgMIC6 C-terminal domain. However, transmembrane microneme proteins in *T. gondii* and *Eimeria* species (F. Tomley personal communication) are unexpectedly partially or completely soluble in the parasites. This biophysical feature is in contradiction with a type I topology predicted from the amino acids sequences of the protein and the identification of tyrosine-based sorting signals in the C-terminal tail. To clarify this point, we performed proteinase K digestion experiments after transient permeabilization. The results unambiguously establish that TgMIC6 adopts a type I membrane topology both during its transport through the ER and Golgi (mic1ko) and when stored in the micronemes.

As previously shown for TgMIC2, both TgMIC6 and TgMIC8 undergo a proteolytic processing event upon secretion, which removes their cytoplasmic tails. The protease, which cleaves at the C-terminus of TgMIC2, has been recently defined as MPP1 (Carruthers et al., 2000). This protease is most likely responsible for the cleavage of TgMIC6 and TgMIC8 and appears to be functionally conserved throughout the Apicomplexa (C. Opitz and D.S., unpublished). In addition to proteolytic processing occurring at the parasite surface, TgMIC6 is N-terminally cleaved during its transport to micronemes. We established here that the processing occurs late in the secretory pathway (Golgi/TGN) and we precisely determined the cleavage site on the protein. The protease responsible for this activity and the biological significance of this intracellular processing remain to be determined. Deletion of the first EGF domain was previously reported to abrogate the processing; however, the absence of the prosequence did not detectably alter sorting or its role as an escorter. Other microneme proteins, TgMIC3, TgMIC5, TgMIC10, and TgM2AP, are also processed during their transport, possibly in the same compartment of the secretory pathway (J. F. Dubremetz, personal communication) (Brydges et al., 2000; Hoff et al., 2001; Rabenau et al., 2001). Interestingly, the TgMIC6 N-terminal cleavage site (VQLS*ETP) strikingly resembles the pro-peptide cleave site for TgM2AP (AQLS*TFL), suggesting that these proteins might be cleaved by the same protease. Further experiments will be required to test this possibility.

In this study, we identified two additional transmembrane proteins containing EGF-like domains. Based on RT-PCR, TgMIC7 and TgMIC9 appeared to be predominantly transcribed in bradyzoites. Their accurate characterization has been hampered by the limited availability of material and inability to cultivate or genetically manipulate this dormant stage. The *TgMIC9* gene is positioned just upstream of the *TgMIC8* locus. The presence of two genes coding for related proteins, being in close proximity on a chromosome has been reported before in the case of the *T. gondii* surface antigens SAG1 and SR1 (Hehl et al., 1997). In *P. falciparum*, the genes coding for MSP2, MSP4 and MSP5 are also arranged in tandem on chromosome 2 (Marshall et al., 1998).

We provide here compelling genetic evidence that TgMIC8 interacts directly or indirectly with TgMIC3, which, by analogy to what is known concerning TgMIC6, strongly suggests that this transmembrane protein may assist in sorting the soluble adhesin TgMIC3 to the micronemes. Biochemical evidence of the existence of this complex based on immunoprecitation has been hampered by the strong tendency

of TgMIC3 to precipitate in detergent extracts. In addition to their role as escorters, TgMIC6 and TgMIC8 are likely to be actively participating as an adhesin complex during the invasion process. The presence of the conserved tryptophan residue in their tails suggests a role in parasite gliding motility. Indeed, as observed for TgMIC2, TgMIC3 was recently shown to redistribute toward the posterior pole of the parasites during invasion (Garcia-Reguet et al., 2000). The posterior capping of TgMIC3 presumably occurs via its interaction with TgMIC8. In contrast to TgMIC6, which does not seem to bind directly to host cells, the presence of a lectin-like domain in TgMIC8 suggests that it could fulfil an adhesive function as well. Interestingly, the adhesive activity of TgMIC3 depends both on the ability of this protein to form homodimers and on the presence of the lectin-like domain (Soldati et al., 2001). The concentration of MIC8GPI in the region of contact between the parasites (Fig. 8B) suggests that TgMIC8 can self-associate. This property does not depend on the presence of TgMIC3 since the same phenomenon of aggregation was observed in mic3ko parasites (M. Meissner, unpublished). Recently, a third complex of microneme proteins, composed of TgMIC2 and TgM2AP, has been characterized (Rabenau et al., 2001). The absence of each one of the two proteins drastically compromises the sorting of the other (C. Opitz and D.S., unpublished; V.B.C., personal communication).

The existence of specific complexes, not only between microneme proteins but also between rhoptry and dense granule proteins suggest that this mode of sorting might be generally and successfully exploited by apicomplexan parasites. In these parasites, the sorting in the secretory pathway is complicated by the existence of multiple distinct secretory organelles, which fulfil specific and crucial tasks in the establishment of intracellular parasitism. These findings build a new and general concept as to how soluble secretory proteins are accurately sorted to their appropriate organelles in these primitive eukaryotes.

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