Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite Toxoplasma gondii

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Abstract
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Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite
Toxoplasma gondii

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Apicomplexan parasites actively secrete proteins at their apical pole as part of the host cell invasion process. The adhesive micronemal proteins are involved in the recognition of host cell receptors. Redistribution of these receptor–ligand complexes toward the posterior pole of the parasites is powered by the actomyosin system of the parasite and is presumed to drive parasite gliding motility and host cell penetration. The microneme protein protease termed MPP1 is responsible for the removal of the C-terminal domain of TgMIC2 and for shedding of the protein during invasion. In this study, we used site-specific mutagenesis to determine the amino acids essential for this cleavage to occur. Mapping of the cleavage site on TgMIC6 established that this process occurs within the membrane-spanning domain, at a site that is conserved throughout all apicomplexan microneme proteins. The fusion of the surface antigen SAG1 with these transmembrane domains excluded any significant role for the ectodomain in the cleavage site recognition and provided evidence that MPP1 is constitutively active at the surface of the parasites, ready to sustain invasion at any time.

Keywords: Apicomplexa/microneme/MPP1 protease/regulated secretion/Toxoplasma gondii

Introduction

The intracellular protozoan parasite Toxoplasma gondii shares with other members of the Apicomplexa a common set of apical structures involved in host cell invasion. The apical secretory organelles, called micronemes, release numerous soluble and transmembrane proteins at the surface of the parasite during invasion (Tomley and Soldati, 2001). TgMIC2, an adhesin secreted during host cell penetration (Carruthers et al., 1999a), belongs to the family of thrombospondin-related anonymous proteins (TRAPs) previously shown to play an essential role in host cell invasion by Plasmodium sporozoites and ookinetes (Sultan et al., 1997; Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000). The C-terminal domain of TgMIC2 was recently demonstrated to functionally replace the equivalent domain of TRAP in Plasmodium (Kappe et al., 1999). TgMIC2 has also been reported recently to form a complex with TgM2AP (Rabenau et al., 2001). Other transmembrane microneme proteins described in T.gondii, such as TgMIC6 and TgMIC8, serve as escortors for the soluble adhesins TgMIC1, TgMIC4 and TgMIC3, respectively (Reiss et al., 2001; Meissner et al., 2002). The stimulation of micronemes discharge is induced by a rise in intracellular Ca²⁺, and can be mimicked by treatment with Ca²⁺ ionophore or ethanol (Carruthers and Sibley, 1999; Carruthers et al., 1999b). Microneme proteins undergo complex proteolytic processing events that have been best documented in T.gondii. TgMIC3, TgMIC5, TgMIC6 and TgM2AP are proteolytically cleaved intracellularly during transport to the micronemes (Brydges et al., 2000; Garcia-Reguet et al., 2000; Rabenau et al., 2001; Reiss et al., 2001). In addition, TgMIC2, TgMIC4, TgMIC6 and TgMIC8 are processed post-exocytosis (Carruthers et al., 2000; Brecht et al., 2001; Reiss et al., 2001; Meissner et al., 2002). Two types of proteases, termed MPP1 and MPP2, have been reported to process TgMIC2 upon release at the parasite surface (Carruthers et al., 2000). Recently, the profile of protease inhibitors that prevent the N-terminal processing of TgMIC4 suggested that MPP2 is likely to cleave this protein as well (Brecht et al., 2001). MPP2-dependent cleavage precedes the MPP1 cleavage, and these maturation events on TgMIC2 have been shown to change the adhesive properties of the protein drastically (Carruthers et al., 2000). The C-terminal cleavage of TgMIC2, TgMIC6 and TgMIC8 is expected to cause the release of the adhesin complexes from the parasite surface. Presumably, this event constitutes an essential step during host cell invasion, ensuring the dissociation between the parasite and the host plasma membrane at the end of the penetration process. Little is known about the sequence requirement, the localization and the characteristics of the protease responsible for this activity.

In this study, we have mapped the site of the MPP1-dependent processing at the C-terminus of TgMIC2, TgMIC6 and a newly characterized microneme protein TgMIC12. The cleavage, which appears to be essential for parasite survival, occurs within the transmembrane domain and does not require sequence information from the ectodomain. The protease is constitutively active at the surface of the parasites and conserved throughout the phylum of Apicomplexa, including in Plasmodium.

Results

The amino acid sequences within the membrane-spanning domain of apicomplexan microneme proteins are conserved

An alignment of the transmembrane and cytoplasmic domains of T.gondii, P.falciparum, P.berghei, Eimeria tenella and Sarcoctysis muris microneme proteins revealed a striking conservation of the amino acid sequences...
within their C-terminal membrane-spanning domain (Figure 1A). In *T. gondii*, TgMIC2 is processed upon secretion, during host cell invasion by an as yet unidentified protease called MPP1 (Carruthers et al., 2000). A very similar processing event occurs on TgMIC6 (Reiss et al., 2001), TgMIC8 (Meissner et al., 2002), TgAMA-1 (Donahue et al., 2000; Hehl et al., 2000) and TgMIC12 (this study), which most probably involves the same protease activity. Taking advantage of the easy accessibility of *T. gondii* to transfection, we have examined the sequence requirements of this processing event. Some of the most conserved residues across the phylum of Apicomplexa (boxed in grey in Figure 1A) were chosen for the site-specific mutagenesis. The constructs generated and analysed in this study are depicted in Figure 1B.

**Point mutations within the transmembrane domain of TgMIC2 and TgMIC6 prevent processing and release of these proteins**

The insertion of a c-myc epitope tag at different positions in the cytoplasmic region of TgMIC2 previously allowed the mapping of sequences relevant for sorting to the micronemes (Di Cristina et al., 2000). A very similar processing event occurs on TgMIC6 (Reiss et al., 2001), TgMIC8 (Meissner et al., 2002), TgAMA-1 (Donahue et al., 2000; Hehl et al., 2000) and TgMIC12 (this study), which most probably involves the same protease activity. Taking advantage of the easy accessibility of *T. gondii* to transfection, we have examined the sequence requirements of this processing event. Some of the most conserved residues across the phylum of Apicomplexa (boxed in grey in Figure 1A) were chosen for the site-specific mutagenesis. The constructs generated and analysed in this study are depicted in Figure 1B.

**Mapping of the C-terminal cleavage site of TgMIC6**

To determine with precision the MPP1 site of cleavage, recombinant parasites expressing TgMIC6Ty were used to purify large amounts of the processed form of TgMIC6...
and to analyse it by mass spectrometry. The mature form of TgMIC6 was isolated from $5 \times 10^9$ freshly released parasites after stimulation of microneme secretion by ethanol and purification by immunoprecipitation using the monoclonal antibody (mAb) BB2 specific for the Ty-1 epitope. The 25 kDa processed product was separated on SDS–PAGE and digested with trypsin. The resulting peptides were analysed by mass spectrometry to identify a peptide whose C-terminus was not generated by trypsin cleavage. A doubly charged peptide ion matching the mass of the peptide EEGSGHAGAIA ($m/z = 499.9$) was detected. This peptide ion was fragmented by collision-induced dissociation and the fragment ions were recorded under conditions optimized for the mass range 500–1200 to confirm the amino acid sequence. The major fragment peaks are assigned to a $b^9$ series with the C-terminal peptide sequence AGAIA (Figure 3). Additionally, the fragments labelled as $y_9$ and $y_{10}$ are consistent with the two N-terminal amino acids EE of the peptide EEGSGHAGAIA.

**MPP1 processing is conserved for micronemal proteins of the Apicomplexa phylum**

The precise determination of the MPP1 cleavage site on TgMIC6 revealed the striking conservation of the amino acid sequence flanking this site in all transmembrane micronemal proteins of the Apicomplexa. In *Eimeria*, EtMIC1 is processed at the C-terminus upon microneme secretion (F. Tomley, personal communication). In *Neospora caninum*, the homologue of TgMIC2, NcMIC2, also lacks the C-terminal domain (Lovett et al., 2000). In *Plasmodium* species, both the redistribution of TRAP from the anterior to the posterior pole and the shedding of the protein by parasites, which presumably involves the C-terminal proteolytic cleavage of TRAP, are crucial for sporozoite gliding motility (Kappe et al., 1999). In a previous study, *P. berghei* TRAP protein was expressed in *T. gondii* (Di Cristina et al., 2000). We compared the expression of PbTRAP and PbTRAP lacking the transmembrane and cytoplasmic tail (PbTRAPTMCD) by transient transfection in *T. gondii*. The evidence for C-terminal processing of the full-length TRAP was obtained by western blot analysis (Figure 4). Consistent with the removal of the TMCD domain, the processed form co-migrated with the PbTRAPTMCD while the deleted mutant remained unprocessed. The C-terminal cleavage of TRAP in *T. gondii* reinforces the notion that this proteolytic event is ubiquitous in the Apicomplexa and the components of the machinery are functionally conserved.

**The minimal amino acid sequence information required for MPP1 cleavage does not include the ectodomains**

The C-terminal domains of TgMIC2 and TgMIC6 carry sufficient information for sorting to the appropriate organelles. When the major surface antigen SAG1 lacking its glycosylphosphatidylinositol (GPI) anchor signal was fused to the transmembrane and cytoplasmic domains of TgMIC2 or TgMIC6, the chimeric products were targeted to the micronemes (Di Cristina et al., 2000; Reiss et al., 2001). These SAG1 fusions exhibited a typical type I membrane topology as demonstrated by transient permeabilization followed by proteinase K treatment (Figure 5A). This type I topology was shown previously to be adopted by wild-type TgMIC6 (Meissner et al., 2002) and TgMIC2 (data not shown). Consequently, the sorting signals present in the C-terminal tail are facing the cytosol and are accessible to the adaptor complex machinery implicated in vesicular trafficking (Hoppe et al., 2000). Analysis of the recombinant parasites stably expressing SAG1TMCDMIC2 by secretion assays revealed that SAG1 is secreted upon...
ethanol stimulation, processed at the C-terminus and released in the cell supernatant in just the same manner as wild-type TgMIC2 (Figure 5B). To demonstrate unambiguously that the same processing event took place on these SAG1 fusions, the point mutations previously introduced in the transmembrane segment of TgMIC2 and TgMIC6 were included in the pSAG1TMCDMIC2 construct. The SAG1TMCDMIC2mut fusion was sorted accurately to the micronemes and, as expected, the C-terminal cleavage of SAG1TMCDMIC2mut was completely abrogated. Identical results were obtained with SAG1TMCDMIC6mut (data not shown). As in the case of MIC2mut, we failed to obtain stable lines expressing SAG1TMCDMIC2mut (Figure 5C). A schematic representation of the products obtained either after proteinase K digestion or upon stimulation of secretion is depicted in Figure 5D.

**TgMIC12 is a novel microneme protein, which also contains targeting signals to the organelle in the cytoplasmic tail**

TgMIC12 is a novel microneme protein recently identified in *T.gondii* and partially sequenced (accession number AAK58479). Antibodies raised against the C-terminus of this protein stained the small apical microneme organelles and detected a product of 50 kDa by western blotting analysis (F.Stavru and D.Soldati, unpublished). The C-terminal region of TgMIC12 exhibited a strong homology to members of the TRAP family (Figure 1) and showed the closest resemblance to the partial sequence of a 70 kDa *S.muris* microneme protein (Tomley and Soldati, 2001). Consistent with previous observations, the fusion of the TMCD domain of TgMIC12 to the SAG1 ectodomain also localized the chimeric protein to the micronemes (Figure 6A). This SAG1TMCDMIC12 fusion is excluded from the parasitophorous vacuole, as illustrated by the absence of co-localization with the dense granule marker, GRA3. In contrast, an inadvertent mutant generated at the extreme C-terminus of SAG1TMCDMIC12 caused a complete mistargeting of the fusion protein to the dense granules and to the parasitophorous vacuolar space as seen by extensive co-localization with GRA3 (Figure 6B). In this SAG1TMCDMIC12* mutant, the last four amino acids, ADMD, have been replaced by the 10 amino acids QTLLKWIRWH (Figure 1B). The reason for this mistargeting is not understood but might involve an incorrect folding of the cytoplasmic tail, which potentially masks the sorting signals and prevents their interaction.
or GPI-anchored proteins in 1998), SAG1TMCDMIC12* is the first example of a SAG1TyTMCDMIC2 shown schematically as the full protein form with cleavage of SAG1TMCDMIC12* occurred constitutively protected from proteinase K treatment and the secreted processed form c-myc epitope tag within the cytoplasmic domain. The smaller form SAG1TyTMCDMIC2mut schematically. The localization of only one parasites expressing SAG1TMCDMIC2 or SAG1TMCDMIC6. The membrane protein topology of the SAG1TMCDMIC2 fusion was determined by partial permeabilization and proteinase K treatment. (B) Western blot analysis of total lysates and ESAs from recombinant parasites expressing SAG1TMCDMIC2 or SAG1TMCDMIC6. The membrane was probed with anti-Ty-1 antibodies. (C) Western blot analysis of recombinant parasites expressing SAG1TMCDMIC2 and SAG1TMCDMIC-mut. The presence of the four point mutations prevents the cleavage of the chimeric protein. The membranes were probed with either anti-myc or anti-Ty-1. (D) The construct SAG1TMCDMIC2 shown schematically as the full protein form with two Ty-1 epitope tags before the transmembrane domain (TM) and the c-myc epitope tag within the cytoplasmic domain. The smaller form protected from proteinase K treatment and the secreted processed form are shown below. The last cartoon shows the construct SAG1TMCDMIC-mut schematically. The localization of only one Ty-1 epitope tag before the TM, as well as the localization of the four point mutations within the TM, is shown.

with the adaptor complex machinery. Although dense granules were described previously as the default pathway taken only by soluble proteins and not by transmembrane or GPI-anchored proteins in T. gondii (Karsten et al., 2000). The proteolytic processing occurring on these proteins and causing their rapid release from the cell surface is anticipated to be an essential step to accomplish the penetration process. In a preliminary experiment, we observed that the insertion of a c-myc tag in close proximity to the transmembrane-spanning domain of TgMIC2 hampered its processing at the C-terminus. Subsequently, we identified by site-specific mutagenesis a few conserved amino acids within the transmembrane domain which were necessary for the cleavage of TgMIC2 to take place. Expression of a non-processed TgMIC2 mutant (MIC2myc2mut, Figures 1B and 2A) appeared to compromise the survival of the parasites as all attempts to generate stable transformants remained unsuccessful. The MPP1 is constitutively active at the plasma membrane of the parasites and does not require the substrate to traffic through the micronemes

Despite the fact that SAG1TMCDMIC12* accumulated in the vacuole, the protein was processed accurately at its C-terminus (Figure 7A). However, in contrast to the proteins targeted to the micronemes, including SAG1TMCDMIC12*, which are only processed after organelle secretion, the cleavage of SAG1TMCDMIC12* occurred constitutively during the intracellular cycle of parasite replication. Since dense granules are constitutively discharging their contents into the vacuole, this result suggested that MPP1-dependent processing occurred either at the parasite surface or in dense granules. To distinguish between the two possibilities, lysates were prepared from extensively washed extracellular parasites and subsequently stimulated for organelle secretion at 37°C for 60 min and analysed by western blot. Both SAG1TMCDMIC12, and SAG1TMCDMIC12 were processed only upon secretion by micronemes and dense granules, respectively (Figure 7B). This result clearly established that the cleavage of SAG1TMCDMIC12* occurred only when the proteins reached the cell surface, and not during storage in the dense granules. Finally, the inhibitory effect of the presence of the five point mutations on SAG1TMCDMIC12mut and SAG1TMCDMIC12mut confirmed that the cleavage observed on the mistargeted SAG1TMCDMIC12* was identical to that occurring on the same protein targeted to the micronemes (Figure 7C).

The subcellular distribution of SAG1TMCDMIC12mut and SAG1TMCDMIC12mut was examined by immunofluorescence on confocal microscopy after transient transfection. As expected, the mutations introduced in SAG1TMCDMIC12mut did not affect targeting to the micronemes, while in the case of SAG1TMCDMIC12mut the absence of cleavage leads to the persistence of the protein at the parasite surface (Figure 7D), instead of an accumulation in the vacuolar space (Figure 6B).

Discussion

Transmembrane microneme proteins are presumed to play a dual essential role during host cell invasion by the Apicomplexa. The adhesin domains present on the luminal side of these proteins or, indirectly, their association with soluble adhesins establish tight and specific interactions with host cell receptors while their cytoplasmic tail presumably is connected to the actomyosin system of the parasite. Only transmembrane proteins are expected to transmit the mechanical force across the plasma membrane and thus to power parasite gliding motion.

Upon stimulation of microneme secretion, some proteins were shown previously to redistribute over the entire surface of the parasites and, as invasion proceeds, these proteins are excluded from the forming vacuole, accumulate at the posterior pole of the parasites, and are shed. This has been documented previously for TgMIC2 (Carruthers et al., 1999a) and TgMIC3 (Garcia-Reguet et al., 2000). The proteolytic processing occurring on these proteins and causing their rapid release from the cell surface is anticipated to be an essential step to accomplish the penetration process. In a preliminary experiment, we observed that the insertion of a c-myc tag in close proximity to the transmembrane-spanning domain of TgMIC2 hampered its processing at the C-terminus. Subsequently, we identified by site-specific mutagenesis a few conserved amino acids within the transmembrane domain which were necessary for the cleavage of TgMIC2 to take place. Expression of a non-processed TgMIC2 mutant (MIC2myc2mut, Figures 1B and 2A) appeared to compromise the survival of the parasites as all attempts to generate stable transformants remained unsuccessful. The
deleterious effect observed due to the absence of cleavage on TgMIC2 underlines the importance of this event for a successful invasion. In the absence of processing, the exocytosed TgMIC2 might create a persistent bridge between the host cell and parasite surface, thus interfering with the proper sealing of the newly formed parasitophorous vacuole and preventing the parasite from replicating inside the vacuole. The introduction of equivalent point mutations on TgMIC6 provoked the same phenotype and was slightly better tolerated by the parasite, but the stable clones could be maintained for only a few passages. TgMIC6 does not bind directly to the host cell surface receptors but functions as an escorter, and presumably, the lack of processing does not lead to such dramatic consequences. Moreover, we have shown previously that TgMIC6 is dispensable, as we could generate mic6ko by homologous recombination but, in contrast, multiple attempts to disrupt the TgMIC2 gene have been unsuccessful so far. This suggests that unlike TgMIC6, TgMIC2 fulfills a more substantial role. In Plasmodium species, by disruption of the genes coding for several members of the TRAP family it was shown that they are a prerequisite for parasite motility and host cell invasion (Sultan et al., 1997; Dessens et al., 1999; Yuda et al., 1999; Templeton et al., 2000).

Previously, fusions of SAG1 to the TMCD domains of microneme proteins TgMIC2 and TgMIC6 established that the sorting signal to the micronemes are confined within the short cytoplasmic tails. In this study, we provide compelling evidence that the short C-terminal domain of these proteins is sufficient to serve as a substrate for MPP1, excluding an essential role for luminal domains, which contain diverse combinations of adhesive motifs (Tomley and Soldati, 2001). The striking and unexpected conservation within the transmembrane domain combined with the results of the mutagenesis suggested to us that MPP1 functions in the hydrophobic environment of the plasma membrane outer leaflet. This hypothesis was confirmed by mapping of the cleavage site of MPP1 within the transmembrane domain combined with the results of the mutagenesis suggested to us that MPP1 functions in the hydrophobic environment of the plasma membrane outer leaflet. This hypothesis was confirmed by mapping of the cleavage site of MPP1 within the membrane-spanning region of TgMIC6. Interestingly, the cleavage site of MPP1 lies within the membrane, apparently beneath the polar head groups of the phospholipids. Enzymes able to cleave polypeptides within the membrane exist and include the site 2 protease (S2P).
(Rawson et al., 1997), which is a zinc metalloprotease that cleaves the sterol-regulatory element-binding proteins (SREBPs) at site 2 within transmembrane segments (Duncan et al., 1998). The release of the signal peptide fragments also requires cleavage in the transmembrane region (Weihofen et al., 2000). Additionally, the famous amyloid β precursor protein, involved in Alzheimer’s disease, is cleaved twice in the middle of the membrane-spanning domain (Citron et al., 1996). The enzyme named γ-secretase is responsible for this activity and is likely to be another member of the family of polytopic membrane proteases, displaying pharmacological properties of an aspartyl protease, with presenilin probably acting as a catalytic component (Esler and Wolfe, 2001). So far, only one metalloprotease activity has been reported in T. gondii (Berthonneau et al., 2000).

The chimeric protein SAG1TMC\textsuperscript{MIC12\#} is mistargeted to the surface of the parasite and, nevertheless, is processed very efficiently at the C-terminus. The introduction of point mutations at the cleavage site in SAG1TMC\textsuperscript{MIC12\#} precisely confirmed that the processing is MPP1 dependent. These results indicate that MPP1 is not stored in micronemes but rather is a plasma membrane constitutively active protease. Previous experiments using cytochalasin D treatment established that MPP1 activity was not concentrated at the posterior pole of the parasites but rather distributed all over the parasite surface (Carruthers et al., 1999a). Interestingly, the substrate does not need to traffic through the micronemes in order to be delivered correctly to the enzyme and processed accurately. SAG1TMC\textsuperscript{MIC12\#} is cleaved very efficiently as soon as the molecule reaches the plasma membrane. Similarly, when expressed in T. gondii, PbTRAP appears to be processed accurately at the C-terminus despite the fact that the protein is mistargeted to the vacuole because the Plasmodium tail of TRAP lacks the complete information for sorting to the micronemes of T. gondii (Di Cristina et al., 2000).

The mistargeting of TgMIC4 to the parasitophorous vacuole in mic6ko (Reiss et al., 2001) allowed us to address the similar question with regard to MPP2-dependent processing. Unlike the situation observed here with MPP1, in mic6ko, TgMIC4 protein accumulated in the vacuolar space but could no longer be cleaved by MPP2 even when microneme secretion was stimulated (M. Reiss, U. Jäkle and D. Soldati, unpublished). It is interesting to note that MPP2 processing is sensitive to cysteine protease inhibitors, and recently a member of the subtilisin-like family of cysteine proteases has been localized to the micronemes of T. gondii (Miller et al., 2001).

The compartmentalization of the substrates (transmembrane microneme proteins) and the enzyme (MPP1) appears to be a safe strategy adopted by the parasite to avoid processing at an inappropriate time, while still preserving an active enzyme ready to respond very quickly to stimulation. The strict conservation of the cleavage site among microneme proteins of different species strongly suggests that MPP1 is functionally conserved among the members of the phylum of Apicomplexa. This broad
functional conservation across species offers a unique opportunity to study some aspects of the events governing *Plasmodium* host cell invasion using *T. gondii* parasites, more easily accessible to rapid transfection and biochemical analysis than the malaria parasites. The presumed crucial role of MPP1 during host invasion and its unique characteristics justify further efforts to identify and characterize this protease as a new potential drug target against a highly relevant group of human and animal pathogens.

**Materials and methods**

**Host cells and Toxoplasma strains growth**

*Toxoplasma gondii* tachyzoites of the RH strain were maintained by growth on monolayers of human foreskin fibroblasts (HFFs) or African green monkey (Vero) cells, grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 5% or 10% fetal calf serum (FCS; Gibco). A clonal isolate of the RH *T. gondii* was used as the recipient strain for the experiments described here. The mi6ko mutant generated in the RH strain was described previously (Reiss et al., 2001).

**DNA constructs**

The cDNA of *T. gondii* TMCDMIC2 was modified by the insertion of a sequence coding for the c-myc epitope tag to generate pMMIC2myc1 and pMMIC2myc2 using a previously described strategy (Di Cristina et al., 2000). The constructs were introduced by site-directed mutagenesis in the transmembrane region of TMCDMIC12 to create pMMIC2myc2mut. The constructs were expressed in *E. coli* and lyzed in RIPA solution (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM EDTA). Polyacrylamide gels (8.5–10%) were run under reducing conditions with 0.1 M dithiothreitol (DTT) in the samples. mAbs 9E10 anti-c-myc; BB2 anti-Ty1 (mouse ascitic fluid; kindly provided by K.Gull, Manchester, UK); T3 4A11anti-TgMIC2 (kindly provided by K.D. Doherty) and rabbit polyclonal antibodies were diluted 1:1000 in PBS, 0.05% Tween-20 and 5% non-fat milk powder. After washing, the nitrocellulose membrane was incubated for 1 h with a peroxidase-conjugated goat anti-mouse antibody (Bio-Rad; 1:3000) and bound antibodies were visualized using the enhanced chemiluminescence (ECL) system (Boehringer).

**Immunoprecipitation**

Parasites (5 × 10^7) were stimulated to secrete and *TgMIC6* was immunoprecipitated according to the protocol described previously (Meissner et al., 2002).

**Indirect immunofluorescence microscopy**

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

**Preparation of secreted microneme proteins**

For the large-scale preparation of excretory secretory antigens (ESAs), ~5 × 10^7 tachyzoites were resuspended in 1 ml of HHE (HBSS containing 10 mM HEPES and 1 mM EGTA). The discharge of micronemes was stimulated by the addition of ethanol to a final concentration of 1.0% and warming to 37°C for 30 min, as previously described (Carruthers et al., 1999b). Cells were removed by centrifugation at 2000 g and the supernatant was kept frozen.

**Mass spectrometry analysis**

In-gel digestion was performed as described (Rosenfeld et al., 1992; Shevchenko et al., 1996) with minor modifications. Briefly, the excised gel plugs were washed with 100 µl of water, 100 µl of 50% acetonitrile and subsequently shrunk in 100 µl of acetonitrile. Modified trypsin (Promega), 12 ng/µl in 40 mM ammonium bicarbonate buffer, was added and incubated overnight at 37°C. Custom-made chromatographic columns packed with either Poros R2 or Poros oligoR3 (50 µm bead size, PerSeptive Biosystems) were used for desalting the supernatant of the trypsic digest. A 20 µg aliquot of Poros sorbent in 5 µl of 75% methanol/1% acetic acid was packed in a constricted GELoader tip (Eppendorf). After equilibration with 40 µl of 1% acetic acid, an R2 column was aligned with an R3 column to trap hydrophilic peptides not bound by the R2 material (Neubauer and Mann, 1999). The sample was loaded, the columns were washed with 20 µl of 1% acetic acid and peptides were eluted separately from the two columns with 1 µl of 75% methanol/1% acetic acid directly into a pre-coated borosilicate nanoelectrospray needle (MDS Protana, Odense, Denmark) for mass spectrometry (MS) analysis. MS analysis was performed on a Q-TOF Pulsar mass spectrometer (PE Siex, Wiesbaden, Germany) equipped with a nano-ESI ion source (MDS Protana, Odense, Denmark). A potential of 900 V was applied to the nanoelectrospray needle. Declustering potential and focusing potential were set to 40 and 180, respectively. For the fragmentation of selected peptides (unit resolution), three different collision energies (22, 27 and 35 V) and two parameter sets (without pulsar function: m/z 100–1900; IQ3 = 9.2; IRD = 0; IRP = 9.2; with pulsar function: m/z 400–1200; IQ3 = 11.2; IRD = 94; IRP = 7.2; IRW = 40; R02 = 7.2) were used. The peptide fragments were labelled according to Bieumann (1988).
Intramembrane cleavage of apicomplexan microneme proteins

Acknowledgements

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References


Page 1585


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