Analysis of the *Sarcocystis neurona* microneme protein SnMIC10: protein characteristics and expression during intracellular development

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Abstract

*Sarcocystis neurona*, an apicomplexan parasite, is the primary causative agent of equine protozoal myeloencephalitis. Like other members of the Apicomplexa, *S. neurona* zoites possess secretory organelles that contain proteins necessary for host cell invasion and intracellular survival. From a collection of *S. neurona* expressed sequence tags, we identified a sequence encoding a putative microneme protein based on similarity to *Toxoplasma gondii* MIC10 (TgMIC10). Pairwise sequence alignments of SnMIC10 to TgMIC10 and NcMIC10 from *Neospora caninum* revealed approximately 33% identity to both orthologues. The open reading frame of the *S. neurona* gene encodes a 255 amino acid protein with a predicted 39-residue signal peptide. Like TgMIC10 and NcMIC10, SnMIC10 is predicted to be hydrophilic, highly alpha-helical in structure, and devoid of identifiable adhesive domains. Antibodies raised against recombinant SnMIC10 recognised a protein band with an apparent molecular weight of 24 kDa in Western blots of *S. neurona* merozoites, consistent with the size predicted for SnMIC10. In vitro secretion assays demonstrated that this protein is secreted by extracellular merozoites in a temperature-dependent manner. Indirect immunofluorescence analysis of SnMIC10 showed a polar labelling pattern, which is consistent with the apical position of the micronemes, and immunoelectron microscopy provided definitive localisation of the protein to these secretory organelles. Further analysis of SnMIC10 in intracellular parasites revealed that expression of this protein is temporally regulated during endopolygeny, supporting the view that micronemes are only needed during host cell invasion. Collectively, the data indicate that SnMIC10 is a microneme protein that is part of the excreted/secreted antigen fraction of *S. neurona*. Identification and characterisation of additional *S. neurona* microneme antigens and comparisons to orthologues in other Apicomplexa could provide further insight into the functions that these proteins serve during invasion of host cells.

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1. Introduction

The apicomplexan parasite *Sarcocystis neurona* is the primary causative agent of equine protozoal myeloencephalitis, the most commonly diagnosed neurologic disease of horses in the United States (Dubey et al., 1991, 2001a; Hamir et al., 1992; MacKay, 1997). The natural life cycle of *S. neurona* utilises the opossum as a definitive host (Fenger et al., 1997; Dubey and Lindsay, 1998) and a variety of small mammal intermediate hosts, including raccoons, nine-banded armadillos, skunks, and cats (Dubey et al., 2000, 2001b; Cheadle et al., 2001a,b; Tanhauser et al., 2001). Horses are infected by ingesting feed and water sources contaminated with *S. neurona* sporocysts (Fenger et al., 1997; Dubey and Lindsay, 1998). In the infected animal, parasites ultimately invade cells within the central nervous system, occasionally causing focal inflammation and neurological damage. Seroprevalence of *S. neurona* in horses is substantial (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997). However, the incidence of equine protozoal myeloencephalitis is considerably lower (MacKay et al., 2000), and the specific factors and events...
contributing to the pathogenesis of this disease are poorly understood.

Apicomplexan parasites are highly polarised cells that are characterised by the presence of an apical complex. This complex is formed by specialised cytoskeletal elements, including the conoid, and three distinct populations of secretory organelles called micronemes, dense granules, and rhoptries. Collectively, the secretory organelles of the apical complex aid in the organisms’ ability to invade host cells and to establish an intracellular environment that is suitable for survival and propagation. Based on protein characteristics and secretion kinetics during cell invasion, microneme proteins appear to be involved in attachment and entry into host cells (reviewed in Dubremetz et al., 1998). Multiple microneme proteins have been identified thus far (reviewed in Tomley and Soldati, 2001), and it is likely that these proteins work in concert to accomplish the critical function of attachment and invasion.

Like other members of the phylum, S. neurona merozoites possess the secretory organelles micronemes and dense granules; however, little is known about the protein contents of these organelles in this species. In an effort to obtain information regarding the molecular composition of S. neurona, an expressed sequence tag (EST) sequencing project was conducted to generate a database of gene sequences from this parasite (Howe, 2001). Herein, we describe a microneme protein from S. neurona, SnMIC10, which was identified from the collection of S. neurona ESTs based on its similarity to Toxoplasma gondii TgMIC10 and Neospora caninum NcMIC10.

2. Materials and methods

2.1. Parasite culture

Merozoites of S. neurona strain SN3 were maintained by serial passage in bovine turbinate cell monolayers, as described previously (Fenger et al., 1997). The SN3 isolate was obtained from a horse from Panama with histologically-confirmed equine protozoal myeloencephalitis (Granstrom et al., 1992) and has been passed extensively in vitro. Upon lysis of the host cell monolayer, S. neurona merozoites were passed twice through 20 G, 22 G and 25 G needles, and filtered through a 3.0 µm Nucleopore® (Whatman) membrane to remove host cell debris. The harvested parasites were counted with a haemocytometer, washed with PBS, and used fresh or frozen, as needed.

2.2. cDNA clone selection

Sarcocystis neurona sequences that were homologous to TgMIC10 were identified by conducting basic local alignment search tool (BLAST) searches of the GenBank dbEST database using the TgMIC10 sequence as the initial query. Selected clones were recovered from the collection of frozen S. neurona EST glycerol stocks, and the cDNA inserts were sequenced using the T3 primer to confirm their identity. To obtain a full-length SnMIC10 cDNA clone, the 5' -end of the SnMIC10 mRNA transcript was amplified from the cSn1 S. neurona cDNA library (Howe, 2001) using a reverse-orientation primer SnMIC10.R (5'-ACCTGACCATACTGTCGTC-3') and the T3 primer of the pBlueScript cloning vector. The resulting PCR product was ligated into the pCR®-2.1 vector (Invitrogen) and sequenced. A BLASTN search of dbEST with the obtained sequence identified an apparent full-length cDNA, SnEST-T4a13c09, which was recovered for further study. The sequence of SnMIC10 is available through GenBank accession #AF532594.

2.3. Sequence analysis

DNA sequencing was performed using the ABI Prism™ BigDye Terminator Cycle Sequencing reaction mix (PE Applied Biosystems). Reactions were purified on CENTRIPUR spin columns (Princeton Separations) and analysed on an ABI 310 Genetic Analyzer (PE Applied Biosystems). Sequences were manually edited and analysed using GeneTool Lite (DoubleTwist®), SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997), ScanProsite (http://www.expasy.ch/tools/scanprosite/), DictyOGlyc (http://www.cbs.dtu.dk/services/DictyOGlyc/) (Gupta et al., 1999), MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988) and BLAST programs (Altschul et al., 1990, 1997) on the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/).

2.4. Recombinant SnMIC10 expression and polyclonal antisera production

The SnMIC10 open reading frame (ORF), without the predicted signal peptide, was PCR amplified using the primers SnMIC10.exp.f (5'-GATCCATATGGCAACACT-TAATGAACTAGGG-3') and SnMIC10.exp.r (5'-GAT-CAAGCTTTCGGCTGCACCTTTTCC-3'), which were designed to incorporate a NdeI and a HindIII restriction site into the 5'- and 3'-end, respectively. The amplified fragment was digested with NdeI and HindIII and ligated into NdeI/HindIII-digested pET-22b expression vector (Novagen), creating the plasmid pETSnMIC10. Escherichia coli strain BL21-CodonPlus (Stratagene) cells were transformed with pETSnMIC10, and a clone that expressed a high level of recombinant SnMIC10 (rSnMIC10) was selected for use. After induction of expression with isopropyl-β-D-thiogalactopyranoside, rSnMIC10 was purified by nickel-column chromatography using the B-PER™ 6xHis Spin purification Kit (Pierce). Purified rSnMIC10 was run in a 12% SDS-PAGE minigel, excised from the gel, and used to immunise one rabbit and one rat for polyclonal antisera production (Cocalico Biologicals, Inc.).
2.5. SDS-PAGE and Western blot analysis

Proteins and whole parasite lysates were suspended in SDS sample buffer, with or without 2-mercaptoethanol, and supplemented with protease inhibitor cocktail composed of 4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma). Proteins were separated in 12% polyacrylamide gels (Laemmli, 1970). For Western blot analysis, proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer (pH 8.3). Membranes were blocked with PBS containing non-fat dry milk, 0.1% Tween 20, and 5% normal goat serum (NGS), and then incubated for 1 h in primary antibody solution. After multiple washes with PBS containing 0.1% NGS and non-fat dry milk, the membranes were incubated with goat anti-rabbit IgG (1:10,000) or goat anti-rat IgG (1:20,000) secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Inc.). After further washing, the membranes were processed for chemiluminescent detection using SuperSignal® substrate (Pierce) or ChemiGlow® substrate (Alpha Innotech, Corp.) and exposed to radiograph film or documented with an imaging system (Alpha Innotech, Corp.).

2.6. Secretion assays

The excretory/secretory antigen fraction from S. neurona was assayed as previously described for T. gondii (Carruthers and Sibley, 1999). Freshly egressed S. neurona merozoites were harvested as described above and washed once with secretion medium (RPMI with 10 mM HEPES + / − 3% FBS). Parasites were resuspended in secretion medium and placed in microfuge tubes for incubation. Merozoites were removed from the secretion medium by centrifugation (two times at 1,000 × g, 5 min, 4°C), and supernatants were either stored at −20°C or immediately processed for Western blot analysis, as described above. To assess protein quantities, spot densitometry was performed on the FluorChem 8800 using AlphaEase FC processing and analysis software.

2.7. Indirect immunofluorescence assay of extracellular and intracellular merozoites

For analysis of extracellular parasites, freshly harvested merozoites were washed with PBS and resuspended in culture medium. Approximately 1.25–2.5 × 10⁶ parasites were spotted on a poly-l-lysine-coated slide and allowed to air-dry. For analysis of intracellular parasites, bovine turbinate host cells were grown on Lab-Tek® II Chamber Slides™ (Nalgene Nunc International) and inoculated with 0.8–1.0 × 10⁴ freshly harvested merozoites. Slides were fixed for 15 min at 4°C in 2.5% formalin-PBS containing 0.25% glutaraldehyde. For permeabilisation of cells, slides were incubated at 0.2% TX-100/PBS for 30 min. Slides were blocked for 30 min with 10% NGS/PBS, followed by incubation for 1 h at room temperature with primary antibody. Slides were rinsed and incubated with goat anti-rat IgG or goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or Texas Red (Jackson ImmunoResearch Laboratories) at a 1:200 dilution. The slides were mounted in Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc.) and examined with a Zeiss axioscope (Carl Zeiss) equipped for phase contrast and epifluorescence microscopy.

2.8. Immunoelectron microscopic (Immuo-EM) examination of intracellular merozoites

For immunoelectron microscopy, infected cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.5 × PBS for 20–30 min on ice. Fixed cells were washed, scraped and centrifuged. The pellet was progressively dehydrated with ethanol and gradually infiltrated with LR-White (London Resin Company) on ice. Finally the sample was transferred to a gelatin capsule and polymerised at 50°C. Ultrathin sections cut from trimmed blocks and recovered on formvar coated grids were placed on 50-μl drops of the polyclonal anti-rSnMIC10 antibody (400:1 dilution) in PBS/2% BSA, followed by incubation with 1:50 goat anti-rabbit IgG conjugated with 10 nm gold (Bbinternational) and a post-labelling wash of PBS/2% BSA containing 0.125 M NaCl. After immunolabelling, sections were stained and analysed with 4% uranyl acetate and 4% lead citrate and observed using a JEO JEM-100 CX II microscope.

3. Results

3.1. Identification of a S. neurona MIC10 homologue (SnMIC10)

To identify potential microneme proteins from S. neurona, the collection of S. neurona ESTs was searched with the sequences of previously identified T. gondii MIC proteins. BLAST searches of dbEST using TgMIC10 as the query identified six contiguous S. neurona ESTs that exhibited moderate similarity to TgMIC10 from T. gondii and NcMIC10 from N. caninum (Expect values < 10⁻⁴). Alignment of the S. neurona sequence contig with the TgMIC10 and NcMIC10 sequences confirmed the sequence similarity (30% sequence identity, 48% similarity) observed in the initial BLAST searches, and it further suggested that the longest cDNA from the contig (SnEST4a16d01) was truncated at the 5’ end (data not shown).

To obtain a full-length cDNA of SnMIC10, the 5’ end of the gene was amplified from the cSn.1 cDNA library using the T3 primer of the pBlueScript cloning vector and a reverse orientation primer (SnMIC10.R) based on the sequence of SnEST4a16d01. The resulting 700-bp ampli-
fication product was cloned into the pCR®-2.1 vector and designated pSnMIC10.5. Sequence analysis of pSnMIC10.5' revealed the expected sequence overlap with SnEST4a16d01, thus demonstrating that these are valid contiguous sequences. A BLAST search of dbEST with the pSnMIC10.5' sequence identified two additional S. neurona ESTs that appeared to be full-length cDNAs of the putative SnMIC10 gene. The longer of these two ESTs, SnEST4a13c09 (GenBank accession # BE635951), was recovered and re-designated pcSnMIC10.

Complete double-strand sequence analysis of pcSnMIC10 revealed a 2,040 bp cDNA insert with a predicted ORF of 765 bp that encodes a protein of 255 amino acids. The 5' untranslated region (UTR) of pcSnMIC10 was 235 bases long, while the 3' UTR was greater than 1 kb in length. Analysis of other SnMIC10 cDNAs revealed several alternative poly-adenylation sites. Amplification of the SnMIC10 genomic locus indicated that the gene lacks introns (data not shown). A hydrophilicity plot of the SnMIC10 amino acid sequence suggested an amino-terminal hydrophobic stretch that corresponds to a predicted signal peptide of 39 amino acids (cleavage between Gly39 and Thr40). Removal of the signal peptide yielded a mature protein of 216 amino acids that has an expected molecular weight of 24.4 kDa. The SnMIC10 primary sequence does not contain any predicted N-glycosylation and O-glycosylation sites.

To determine the level of sequence conservation in the MIC10 orthologues, a multiple sequence alignment was generated using the presumptive mature forms (i.e. less the predicted N-terminal signal peptides) of SnMIC10, TgMIC10 and NcMIC10. This analysis revealed 33% sequence identity between SnMIC10 and TgMIC10 and 27% identity between SnMIC10 and NcMIC10 (Fig. 1). Relative to TgMIC10 and NcMIC10, the mature SnMIC10 contains a 68 amino acid extension on the amino-terminal end of the protein and an additional seven residues on the carboxyl-terminus. Like its homologues, SnMIC10 is devoid of cysteine residues or predicted adhesive domains, is expected to be predominantly alpha-helical in nature, and is rich in charged amino acid residues thereby resulting in an overall hydrophilic protein. In contrast to the nine diglutamic acid repeats observed in TgMIC10 (Hoff et al., 2001), only two of these repeated elements are present in SnMIC10.

3.2. Characterisation of SnMIC10

To produce reagents for the study of native SnMIC10, a recombinant form of the protein (rSnMIC10) was expressed in E. coli and used to immunise animals for polyclonal antisera production. Western blot analysis using the resulting anti-rSnMIC10 sera revealed a single protein in S. neurona merozoites that migrated at approximately 24 kDa (Fig. 2), consistent with a calculated Mr of 24.4 kDa for the mature SnMIC10. Additionally, SnMIC10 was found in the aqueous phase of a TX-114 partitioning assay (Fig. 2), as expected from the predicted hydrophilic nature of the protein.

The clear homology between SnMIC10 and the MIC10 proteins from T. gondii and N. caninum suggested that SnMIC10 is a microneme protein of S. neurona. Immunofluorescent labelling with the anti-SnMIC10 serum showed a polar staining pattern in extracellular merozoites, consistent with the apical location of micronemes (Fig. 3). To obtain definitive localisation of SnMIC10, S. neurona merozoites were examined by immuno-EM. As shown in Fig. 4, the majority of the immunogold label decorated the micronemes of intracellular merozoites. A small amount of

![Fig. 1. Multiple sequence alignment of the mature proteins (i.e. less the predicted N-terminal signal peptides) showed 31 and 27% identity between SnMIC10 and the TgMIC10 and NcMIC10, orthologues, respectively. Numbers indicate the residue position. Capital letters in the consensus line indicate absolute amino acid conservation. Symbols indicate agreement in two of the three sequences and high consensus value (similar amino acid) in the third sequence substitution. Lower case letters indicate that the third sequence substitution is low consensus (i.e. dissimilar residue).](image-url)
immunogold label was observed in small vesicles anterior to the nucleus, and this likely represents SnMIC10 in the Golgi apparatus. Collectively, these data demonstrated that SnMIC10 is a microneme protein of *S. neurona* merozoites.

### 3.3. Secretion of SnMIC10

To assess whether SnMIC10 is a component of the *S. neurona* excreted/secreted antigen fraction, freshly egressed merozoites were incubated in medium, and the supernatants of these incubations were examined by Western blot for the presence of the protein. As shown in Fig. 5, SnMIC10 was present in the supernatant, consistent with the active secretion of the protein by extracellular parasites. Secretion was dependent on temperature (Fig. 5B), with increased amounts of SnMIC10 detected in supernatants from parasites incubated at 37°C. To determine the quantity of SnMIC10 present in the supernatants, the digital Western blot images captured with the CCD camera-equipped FluorChem 8800 were analysed by spot densitometry using AlphaEase FC processing and analysis software. These analyses indicated that approximately 15–18% of the total cellular SnMIC10 was present in the supernatants (Figs. 5A,B). The secreted SnMIC10 co-migrated in SDS-PAGE with the non-secreted form in lysed merozoites, thus indicating that SnMIC10 did not undergo proteolytic processing during secretion. To monitor for cell lysis, the quantity of surface antigen SnSAG4 or parasite actin in the supernatants was similarly determined. Approximately 6% of the total SnSAG4 (Fig. 5A) or 9% of total parasite actin (Fig. 5B) was detected in the supernatants, demonstrating that some inadvertent cell lysis occurred during the incubation. However, the amount of cell lysis was insufficient to account for the quantity of SnMIC10.

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**Fig. 2.** Western blot analysis of a TX-114 partitioning assay showed that SnMIC10 is present in the aqueous (soluble protein) fraction of *S. neurona* merozoite lysates. A membrane-associated control (SnSAG1) partitioned into the detergent fraction. I = Insoluble, A = Aqueous, D = Detergent.

**Fig. 3.** Immunofluorescent labelling of permeabilised extracellular *S. neurona* merozoites with rat anti-SnMIC10 and rabbit anti-SnSAG1 showed polar localisation of SnMIC10. Proteins were visualised with anti-rat conjugated to FITC and anti-rabbit conjugated to Texas Red. Parasite nuclei were stained using the DNA intercalating dye DAPI.

**Fig. 4.** Immuno-EM analysis of intracellular *S. neurona* merozoites revealed microneme localisation of SnMIC10. The parasite membrane is indicated with black arrowheads. The white box delineates the region enlarged for the inset. The white arrowhead in the inset indicates a gold-labelled microneme. Parasites were fixed with formaldehyde and embedded in LR-White resin to preserve epitopes. Ultrathin sections were labelled with rabbit anti-SnMIC10 and subsequently with gold-conjugated, goat anti-rabbit. Sections were visualised with lead citrate and uranyl acetate staining. HC = host cell, Ap = Apical end, N = parasite nucleus.
observed in the medium, thereby indicating that the protein is actively secreted by extracellular merozoites.

3.4. SnMIC10 expression during intracellular growth

To assess whether SnMIC10 is constitutively expressed throughout endopolygeny and to determine the location of the protein during parasite propagation, immunofluorescent labelling of SnMIC10 was performed on intracellular parasites. This analysis revealed SnMIC10 label at one pole of intracellular parasites at 16 h post-inoculation (Fig. 6A). At approximately 4 days post-inoculation when multiple different developmental stages were present, early- to mid-stage schizonts showed minimal SnMIC10 labelling with only a small, condensed spot observed on one or both poles (Fig. 6B). An increase in SnMIC10 staining was seen in the mid- to late-stage schizonts, with the label dispersed in a punctate pattern throughout the body of the schizont (Fig. 6C). In the final stages of schizont maturation when cytokinesis occurred, the SnMIC10 label became organised to the anterior pole of each individual merozoite (Fig. 6D).

4. Discussion

Micronemes are secretory organelles of the Apicomplexa that are important for attachment and invasion of host cells by these intracellular parasites (Dubremetz et al., 1998). Since the process of host cell invasion appears to be fairly conserved, members of the Apicomplexa are expected to share numerous microneme protein homologues. However, the various species of Apicomplexa occupy a broad range of parasitological niches (e.g. hosts and cell types), and this undoubtedly has necessitated some divergence in the microneme contents of the different members of this phylum. Although there is significant information regarding the composition of micronemes in several members of the Apicomplexa (e.g. T. gondii), only two microneme proteins have been identified thus far in the genus Sarcocystis (Tomley and Soldati, 2001). In the present study, we have identified and characterised a microneme protein of S. neurona merozoites, which has been designated SnMIC10. Identification of the SnMIC10 gene was accomplished by searching the S. neurona EST database with the sequence of TgMIC10 from T. gondii. The SnMIC10 protein shares only about 30% sequence identity with TgMIC10 and the MIC10 homologue from N. caninum, NcMIC10. However, the general protein characteristics of the MIC10 orthologues are predicted to be similar, suggesting that this protein has a relatively conserved function in all three parasite species.

The homology of SnMIC10 with TgMIC10 and NcMIC10 suggested that SnMIC10 is a microneme protein of S. neurona merozoites, which was confirmed by immunofluorescence and immuno-EM analyses. As expected for a microneme protein, SnMIC10 was found to be part of the S. neurona excretory/secretory antigen fraction, as seen previously for the microneme contents of other Apicomplexa (Carruthers et al., 1999; Brydges et al., 2000; Bumstead and Tomley, 2000). Similar to what was observed for TgMIC10 (Hoff et al., 2001), the secreted SnMIC10 co-migrated in Western blots with SnMIC10 from whole merozoite lysate, indicating that the protein is not processed during secretion. This contrasts with numerous other microneme proteins, which have been shown to undergo proteolytic processing upon release from the parasite (Achbarou et al., 1991; Brydges et al., 2000; Lovett et al., 2000; Brecht et al., 2001). Similar to what has been seen for microneme proteins of other Apicomplexa (Bumstead and Tomley, 2000; Lovett et al., 2000; Carruthers et al., 1999; Brydges et al., 2000), the secretion of SnMIC10 was shown to be temperature sensitive, with a marked increase in SnMIC10 release observed at 37°C when compared to both 0 and 25°C.

Microsequencing of TgMIC10 demonstrated that Asp^{59} was the amino-terminal residue of the mature protein, thus indicating that the signal peptide is 19 amino acids longer than predicted since this protein does not undergo post-translational processing (Hoff et al., 2001). The sequence alignment of SnMIC10 with TgMIC10 and NcMIC10
revealed that a 68-residue amino-terminal extension exists on the *S. neurona* orthologue. The amino-terminus of mature SnMIC10 was not verified by microsequencing, so we cannot rule out post-translational processing of SnMIC10 that would yield a protein equal in length to TgMIC10 and NcMIC10. However, migration of SnMIC10 in SDS-PAGE was consistent with the expected Mr for the described primary sequence, suggesting that the 68-amino acid extension is part of the mature protein. It is unknown whether there is any functional significance to the amino-terminal extension.

*Sarcocystis neurona* propagates by endopolygeny, a developmental process similar to schizogony in which a single parasite gives rise to multiple daughter merozoites. During endopolygeny and schizogony, DNA synthesis is dissociated from cell division, thereby producing a large polyploidy cell. In the final stages of development, cytokinesis occurs to form the individual merozoites. Immunofluorescent analysis of intracellular *S. neurona* revealed that expression of SnMIC10 is temporally regulated during endopolygeny. The SnMIC10 label was maintained at one pole of intracellular parasites for up to 16 h post infection. As the parasites progressed through endopolygeny, the SnMIC10 label decreased, thus suggesting that SnMIC10 and possibly other microneme proteins are not expressed during this phase of the cell cycle. Interestingly, a small spot of labelling was always seen on one or both poles of the schizont, and this most likely represents the remnant micronemes from the primary merozoite that invaded the host cell. As the schizonts matured, SnMIC10 production was apparently up-regulated, and the protein label appeared in a dispersed, punctate pattern throughout the body of the developing schizont. These spots are presumably the nascent micronemes of the newly-forming daughter cells. At the completion of cytokinesis, the SnMIC10 label had become located at the apical end of each merozoite. The down-regulation of SnMIC10 observed during endopolygeny is concordant with the loss of micronemes seen by ultrastructure analysis of *Sarcocystis* spp. schizonts (Dubey et al., 1989), and it is predictable for proteins that are not needed during the prolonged and complex intracellular development of these

Fig. 6. Immunofluorescent labelling of intracellular *S. neurona* with rat anti-SnMIC10 and DAPI nuclear stain at 16 h post infection showed polar localisation of SnMIC10 label (arrow) (A). Early to mid-stage schizonts (outlined by arrowheads) contained a small spot of SnMIC10 label on one or both poles (arrow) (B); mid to late stage schizonts (outlined by arrowheads) showed diffuse punctate labelling of SnMIC10 (C), and merozoites in mature schizonts exhibited polar localisation of SnMIC10 (D). Parasites were visualised with goat anti-rat FITC, and parasites in panels B–D were co-labelled with rabbit anti-SnSAG1 and visualised with goat anti-rabbit Texas Red. HCN = host cell nucleus.
parasites. Somewhat similar to what was observed for SnMIC10, the microneme protein EtMIC2 of *Eimeria tenella* can be weakly detected in the cytoplasm of immature schizonts, but staining becomes more intense and concentrated to the apical end of the newly-formed merozoites as the schizonts mature (Tomley et al., 1996). In contrast to the aforementioned organisms that undergo endopolygeny/schizogony, the microneme proteins MIC1, MIC2, and MIC3 are present throughout intracellular development of *T. gondii* (Achbarou et al., 1991), which is consistent with the continued existence of micronemes in the developing daughter cells during endodyogeny (Hu et al., 2002).

The contents of the micronemes are known to be heterogeneous, and each of the assorted microneme proteins likely serves a different function to help accomplish host cell invasion. A number of microneme proteins have been shown to possess various adhesive domains, including integrin I domains, apple domains, EGF-like domains, and thrombospondin type I regions (Tomley and Soldati, 2001). These domains likely provide the parasites with an array of host cell-attachment capabilities. The absence of adhesive domains in the MIC10 orthologues and the lack of host cell binding by TgMIC10 (Hoff et al., 2001) suggest that this microneme protein is not involved in a receptor-ligand interaction with a host cell surface molecule during the invasion process. Unfortunately, the primary sequences of the three MIC10 orthologues provide little indication of this protein’s function, so the specific role served by MIC10 during infection remains unknown. However, it can be assumed that regions of sequence conservation are likely important for a function(s) that is shared by all three MIC10 orthologues. The nine diglutamic acid repeats and the RK(R/Y)HEEL repeat sequences seen in TgMIC10 are not well conserved in the *S. neurona* homologue nor are they prevalent in the NcMIC10 orthologue (Hoff et al., 2001). Consequently, these repeat elements may be necessary only for a task that is specific to TgMIC10. Both *T. gondii* and *N. caninum* are amenable to molecular genetic manipulations, and the generation of MIC10 deficient mutants may provide some insight into how this protein serves as a virulence factor for this group of important pathogens.

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