Molecular characterisation of a major 29 kDa surface antigen of *Sarcocystis neurona*∗

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Abstract

A gene encoding a major 29 kDa surface antigen from *Sarcocystis neurona*, the primary causative agent of equine protozoal myeloencephalitis (EPM), was cloned, sequenced, and expressed as a recombinant protein. A cDNA library was prepared in the expression vector lambda ZAP from polyA + mRNA isolated from *S. neurona* merozoites cultivated in vitro. Random sequencing of 96 clones identified a clone of an abundant transcript having a translated amino acid sequence with 30% identity to the 31-kDa surface antigen of *Sarcocystis muris* cyst merozoites. Southern blot analysis indicated that the corresponding gene exists in low copy number within the *S. neurona* genome, but RNA blot analysis and other data indicated that the gene transcript is highly abundant. The sequence of the cDNA clone encoded an open reading frame specifying a polypeptide of 277 amino acids with a predicted size of 28.7 kDa. The deduced amino acid sequence displayed a hypothetical N-terminal signal peptide sequence followed by a polypeptide containing 12 cysteines. The coding region of the cDNA insert was subcloned into the expression vector pET14b, and a fusion protein expressed. The recombinant polypeptide was recognised by mAb 2A7 and mAb 1631, directed against a 29 kDa native protein found on the surface of cultured merozoites. Antibodies in serum and cerebrospinal fluid from a horse with EPM recognised a 29 kDa native protein of *S. neurona* merozoites and the 29 kDa recombinant protein. This *S. neurona* surface antigen is named SnSAG1. © 2001 Published by Elsevier Science Ltd. on behalf of Australian Society for Parasitology.

Keywords: Apicomplexan; *Sarcocystis neurona*; Surface antigen 1-related sequence; Merozoite; Equine protozoal myeloencephalitis

1. Introduction

Equine protozoal myeloencephalitis (EPM) is a well-recognised neurological disease of horses in North and South America caused primarily by *Sarcocystis neurona* (Dubey et al., 1991). The horse is an aberrant host for *S. neurona*, however, since it does not form infectious sarcocysts in the horse. Rather, it naturally cycles between the Virginia opossum (*Didelphis virginiana*) and either the striped skunk (*Mephitis mephitis*) (Cheadle et al., 2001b) or the nine-banded armadillo (*Dasypus novemcinctus*) (Cheadle et al., 2001a; Tanhuaser et al., 2001). The parasite has been cultured from central nervous system (CNS) lesions of horses from several different locations in the USA, and these isolates show only minor differences when compared by current morphologic, immunologic, and DNA sequence-based tests (Marsh et al., 1997b, 1999; Tanhuaser et al., 1999; Speer et al., 2000; Rosenthal et al., 2001). At some point during the infection in the horse, *S. neurona* travels to the brain and spinal cord, where merozoite stages of this parasite replicate and cause disease. Other species of *Sarcocystis* have been described as disseminating in the intermediate host via the bloodstream (Fayer, 1979; Fayer and Leek, 1979; Fayer and Dubey, 1984), and when a foal with severe combined immune deficiency was infected with *S. neurona* sporocysts derived from a feral opossum, *S. neurona* merozoites were isolated from the circulatory system (Long, MT, et al., unpublished data). During infection, the merozoite is exposed to the host’s immune system, thus it is possible that such exposure to parasite antigens under the right conditions may elicit a protective immune response. Cloning and expressing genes that encode *S. neurona* surface antigens is a useful first step toward preparing specific immunologic reagents to study the pathogenesis of EPM in the horse. Presently, whole

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merozoites of *S. neurona* are used as antigens in the immuno-
noblot to demonstrate the presence of *S. neurona*-specific
antibodies in infected horses (Granström et al., 1993), and
native antigens recognised by such sera may be candidates
for further characterisation. Here, we report the molecular
characterisation of a 29 kDa protein on the surface of *S.
neurona* merozoites which we termed SnSAG1.

2. Materials and methods

2.1. Preparation of *S. neurona* merozoites and derived
reagents

Isolates UCD1 and SnFL1 of *S. neurona* were cultured
and merozoites released from host cells as previously
described (Ellison et al., 2001). The SnUF1 isolate, recovered
from the spinal cord of a horse with clinical EPM and
propagated in BT cells, was the gift of R.J. MacKay. All
studies were done with merozoites of isolate UCD1 except
where noted. Controls included studies with antigens and
nucleic acids derived from *Toxoplasma gondii* (RH) (Fren-
kel, 1953), *Neospora caninum* (Ne-1) (Dubey et al., 1988),
*Neospora hughesi* (Oregon) (Dubey et al., 2001), and *Sarco-
cystis falcata* (ATCC No. 50701) propagated in in vitro
culture. Total RNA was extracted with RNAzol and purified
as per the manufacturer’s recommendations (Tel-Test Inc.).
Polyadenylated RNA (polyA-RNA) was recovered from
total RNA via binding to oligo dT linked to magnetic
beads (Promega) as per the manufacturer’s recommenda-
tions.

2.2. Immunofluorescence assay (IFA) and immunogold
labeling

Parasites for fixed IFA were harvested and washed with
phosphate buffered saline (PBS), applied to the slide by
cytospin, and fixed with methanol. Slides were blocked
with the application of 100 µl dilute goat serum in PBS
(1:400) for 30 min at 37°C to block non-specific binding.
Slides were washed in PBS containing 0.5% Triton × 100
(PBST) for 5 min with three changes of buffer and drained
of excess fluid. Monoclonal antibodies (2A7, 1631 or
isotype control, described below) were applied at ~10 µg/
ml in PBS and incubated for 1 h at 37°C followed by wash-
ing as above. Fluorescein isothiocyanate- (FITC)-labelled,
goat-anti-mouse secondary antibodies (Sigma) were applied
and parasites were observed by light and epifluorescent
microscopy. For live IFA parasites were harvested, washed
twice in media (Dulbecco modified Eagle’s medium
(DMEM) from Media Tech, Inc. supplemented with 10
mM HEPES buffer and normal goat serum, 1:400), resus-
pended in media containing mAb 1631 or isotype control at
~10 µg/ml, incubated 1 h, and washed twice by resuspen-
sion in media followed by centrifugation. Parasites were
then resuspended in media containing FITC-labelled, goat-
anti-mouse secondary antibodies (Sigma), incubated 1 h,
2.5. Expression of fusion protein

*Sarcocystis neurona* cDNA was used as the template for PCR amplification of the coding region of the clone of interest by PCR using oligonucleotides 5'-ACG AGG ATC CGA TGA CGA GGG CGG TGC TGC TG-3' and 5'-ACG AGG ATC CCA CGG CAG GAT TAG CAA AAG TGC-3' as forward and reverse primers, respectively. These primers had BamHI recognition sequences appropriately positioned to subclone the open reading frame of the full SnSAG1 coding region into pET14b. The Taq amplification product from a reaction cycle of 93°C, 5 s, 65°C, 30 s, 72°C, 1 min repeated 30 cycles was isolated by agarose gel electrophoresis and cloned into the TOPO vector (Invitrogen).

The sequence of the amplified SnSAG1 coding region cloned in the TOPO vector was verified and subcloned into the BamHI site of the expression vector pET14b-antibody (Novagen). The pET14b expression construct was transfected into host BL21 and production of recombinant SnSAG1 (rSnSAG1) was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the bacteria were in mid-log growth phase (A600 = 0.6). Growth at 37°C was continued for 2 h before harvesting the cells. The fusion protein was detected on immunoblots using an anti-His tag mAb (Novagen). Induced cells were collected by centrifugation at 3000 × g at 4°C for 20 min. Cells were washed once with PBS and inclusion bodies purified as described by the manufacturer (Novagen) except that the cells were disrupted by passing them through a French press (15,000 psi). Inclusion bodies were solubilized in TN buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 6 M urea for 40 min at 4°C. Insoluble material was removed by centrifugation at 8700 × g for 30 min, at 5°C. The Histagged, rSnSAG1 was purified by affinity chromatography on a His-bind column (Novagen).

2.8. Nucleic acid blotting and analysis

*Sarcocystis neurona* merozoites were harvested from cultures at 12 days post-infection (P.I.) (Ellison et al., 2001), and DNA was extracted in lysis buffer (50 mM Tris–HCl, pH 8.0, 62.5 mM EDTA, pH 8.0, 2.5 M LiCl, 4% Triton × 1000) followed by phenol–chloroform extraction and ethanol precipitation. The precipitated DNA was pelleted by centrifugation, washed in 80% ethanol, air-dried, and dissolved in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (TE buffer). DNA was extracted from *T. gondii*, *N. caninum*, and *S. falcatula* in a similar fashion. DNA (5 μg) aliquots were digested with EcoRI and fragments separated by electrophoresis on a 0.8% (w/v) agarose gel. Southern blotting was performed by capillary transfer onto nylon membranes following acid depurination, alkaline denaturation, and neutralisation steps. For Northern analysis, RNA was produced as described above and was analysed by agarose/formaldehyde gel electrophoresis. The RNA, 1 μg, was transferred to a nylon membrane. After prehybridisation (2 × SSC containing salmon sperm DNA) for 2 h at 60°C, a probe prepared from the open reading frame of SnSAG1 was 32P-labelled by the random primer method (Feinberg and Vogelstein, 1984) and added to the hybridisation buffer for 24 h at 60°C. The membrane was washed in...
Fig. 1. Immunofluorescent assay of the binding of mAbs 2A7 and 1631 to merozoites of *Sarcocystis neurona*. Binding of mAb 2A7 to a methanol-fixed merozoite preparation as detected by fluorescein-labelled, goat anti-mouse antibody under white light illumination (A); and under ultraviolet illumination (B). Binding of mAb 1631 to live merozoites as detected by similar assay under white light illumination (C); and under ultraviolet illumination (D). The lack of binding of isotype control mAb to live merozoites as demonstrated by similar assay under white light illumination (E); and under ultraviolet illumination (F).

0.2×SSC, 0.2% SDS at 55°C. Autoradiography was performed with enhancing screens for 8 days at −80°C. The probe was also used to hybridise blots of DNA from *S. neurona*, *T. gondii*, *N. caninum*, and *S. falciparum*. Bands were visualised by autoradiography.

3. Results

Monoclonal antibodies 1631 and 2A7 bound strongly to *S. neurona* merozoites as viewed by immunofluorescence microscopy. The binding of mAb 2A7 to fixed merozoites is shown in Fig. 1A, B, and similar results were found for mAb 1631 by live IFA (Fig. 1C, D). As shown in Fig. 1C, D many of the merozoites in the developing rosette bound less of the mAb 1631 than free merozoites. No binding was seen with the mAb isotype control (Fig. 1E, F). Merozoites of *S. falciparum* examined in a similar assay did not bind mAb 1631 (data not shown). Post-embedding immunogold labelling followed by transmission electron microscopy was used to confirm binding of these mAb to the parasite surface. Gold-labelled, anti-mouse antibodies bound the surface of the embedded parasites treated with either mAb 2A7 or mAb 1631. The immunogold labelling of merozoites treated with the latter mAb is shown in Fig. 2A, B; data from mAb 2A7 are not shown. Isotype control mAb did not label the merozoites (Fig. 2C). The mAbs 1631 and 2A7 also bound a 29 kDa antigen on immunoblots of SDS-polyacrylamide gel electrophoresis (PAGE) separated native antigens derived from cultured *S. neurona* merozoites (Fig. 3). Merozoite proteins separated by SDS-PAGE were blotted and reacted with various antibody preparations. The immunodominance of antigen(s) at ~29–30 kDa in a mouse immunised with merozoites suspended in adjuvant was evident (Fig 3A, lane 1) when compared with a large number of other less intense bands from other merozoite antigens recognised by this hyperimmune polyclonal serum. Binding of mAb 2A7 and mAb 1631 to an antigen at 29 kDa was specific as seen in Fig. 3B, lanes 2 and 4, respectively. When the antigen preparation was reduced by heating in the presence of β-mercaptoethanol, binding of both mAbs was greatly reduced or abolished (Fig. 3B, lanes 1 and 3). Neither of these mAbs bound to host cell antigens (Fig. 3B, lanes 8–10) nor to *N. caninum* or *T. gondii* tachyzoite antigens on immunoblots (data not shown).

Clones from a cDNA library constructed from the mRNA of *S. neurona* merozoites were randomly sequenced. Partial sequence of one clone containing a 687 bp insert when
translated into an amino acid sequence had 30% identity to the major surface antigen of *S. muris* cyst merozoites, SmSAG1. This partial clone was used as a probe to obtain a full-length copy of the coding region of the gene. Screening the library by hybridisation with this probe demonstrated that ~10% of clones screened shared sequence similarity to this clone. Analysis of seven positive clones revealed that many of the cDNA inserts were partial clones of similar length (~0.83 kb), and they shared a common restriction map (not shown). One larger clone had an extended 5'-untranslated region with a total insert size of 1232 bp (Fig. 4A). The sequence of this longer clone contained a 5' non-coding region of 72 bp, a single long open reading frame of 831 bp, and a 330 bp non-translated region. When the probe was hybridised to a blot of total RNA isolated from *S. neurona* merozoites, one strong band was detected at 1.5 kb (Fig. 5A). It thus appears that the cDNA clone we have sequenced contains most of the major mRNA transcript. A blot of EcoRI-cleaved genomic DNA hybridised with this probe yielded a single band at approximately 1.2 kb (Fig. 5B). This probe did not hybridise to bovine (host cell) DNA or the DNA of *S. falciparum, N. caninum,* or *T. gondii* (data not shown). We have used reverse transcriptase- (RT)-PCR data to confirm the presence of SmSAG1 in four isolates of *S. neurona* (data not shown). The other closely related *Sarcocystis* spp. (e.g. *S. falciparum* and 1085-like isolates) remain to be tested, but the same primers did not amplify a product when genomic DNA from *T. gondii* or *N. hughesi* were used.

Fig. 4. SmSAG1 cDNA sequence with translated peptide sequence and alignment with *Sarcocystis muris* SAG1. (A) A putative signal peptide is underlined, as is the Ala at residue 255, the site of a predicted GPI anchor. The arrowhead marks the position of the intron shown in (Panel C). The asterisk notes the termination codon for translation. All cysteine residues are in shaded boxes. (B) Most cysteine residues (shaded boxes) are conserved in the alignment with SmSAG1 (GenBank Accession No. M76496). (C) Sequence of the 128 bp intron present in the genomic DNA copy of SmSAG1.
The ATG codon at the beginning of the reading frame was preceded by two in-frame termination codons (underlined) at positions −39 and −66 in the 5’ upstream region (Fig. 4A). The 828 bp reading frame encoded a polypeptide of 276 amino acids with a calculated Mr of 28,328 Da and an isoelectric point (pl) of 7.48. The predicted protein contained a membrane protein with a cleavable signal peptide and one transmembrane spanning region near its C terminus (McGeoch, 1985; Hartmann et al., 1989). It had a potential cleavage site at Ala 255 (underlined) suggesting a possible GPI anchor at this position (Eisenhauber et al., 1999). The sequence had 12 cysteine residues, 10 of which were conserved when compared with SnSAG1 (Fig. 4B), and nine were conserved when the comparison was extended to N. caninum SAG1 (NcSAG1, GenBank Accession No. AF132217, alignment not shown).

Examination of the genomic DNA encoding SnSAG1 by PCR indicated that the genomic sequence was slightly larger than that of the cDNA. Sequence analysis indicated the presence of a single 128 bp intron located at position 406/407 (arrow in Fig. 4A) in the coding region of the mature mRNA. The sequence of the intron is presented in Fig. 4C. Over-expression of SnSAG1 in E. coli using pET14b produced a recombinant protein, with an N-terminal sequence containing six His residues that is slightly larger than the native antigen at 29 kDa. The rSnSAG1 was recognised on immunoblots (Fig. 6A) by His-tag mAb (lane 1), mAb 2A7 (lane 2), CSF from a horse with clinical EPM (lane 3), but not by serum from European horses never exposed to S. neurona (lane 4). Monoclonal antibodies 2A7 and 1631 bound to epitopes on rSnSAG1 that were sensitive to reduction with sulphydryl reducing agents, but amounts of β-mercaptoethanol twice normal were necessary to eliminate the epitope from rSnSAG1 on Western blots (Fig. 6B, lanes 1–4 and Fig. 7). A portion of the rSnSAG1 appeared to form a dimer on some gels which also disappeared under reducing conditions (Fig. 6B, lanes 1–4, 7–8). IgG1 control antibody did not bind to rSnSAG1 either non-reduced or reduced (Fig. 6B, lanes 9–10). Serum from a horse with clinical EPM bound to rSnSAG1 in a fashion similar to the mAbs 2A7 and 1631 (Fig. 6B, lanes 11–12). When β-mercaptoethanol was increased in the sample buffer from 5 to 10%, the amount of detectable rSnSAG1 was greatly reduced (Fig. 7).

4. Discussion

Whole cultured merozoites of S. neurona comprise the antigen currently used on immunoblots to demonstrate the presence of antibodies in infected horses (Granstrom et al., 1993). Antibodies that are considered diagnostic for exposure and consistent with disease differ among various laboratories, but those that bind to antigens on blots at approximately 29–30 and 19 kDa are prime candidates where issues of cross-reacting antibodies are resolved (Marsh et al., 1996; Rossano et al., 2000). The sera of infected horses react strongly to the band at 29 kDa suggesting that this is an immunodominant antigen (Liang et al., 1995).
1998). More specifically, this antigen designated here as SnSAG1 is also recognised on immunoblots when probed with intrathecal antibodies from horses naturally infected with S. neurona and exhibiting signs of EPM. Monoclonal antibodies 1631 and 2A7 raised against whole merozoites and polyclonal antisera raised against rSnSAG1 each bind both the native merozoite protein of 29 kDa and the purified, non-reduced rSnSAG1 on blots of proteins separated by SDS-PAGE. Immunofluorescent antibody assay and post-embedding immunogold labelling studies using mAb 2A7 and mAb 1631 localised SnSAG1 on the surface of S. neurona merozoites.

The gene encoding SnSAG1 is found on a 1.2 kb EcoR1 fragment of genomic DNA, but the copy number and genomic DNA sequence are yet to be determined. There is an abundance of SnSAG1 mRNA, as suggested by a very strong band on Northern blots and by being found at a frequency of ~10% in our cDNA library. The latter observation is further supported with data provided from the 1700 sequence tags of S. neurona in the dbEST at the National Center for Biotechnology Information (NCBI) provided by D.K. Howe. When sequence from SnSAG1 is compared with the dbEST database using the BLAST algorithm, the report identifies 101 sequence tags sharing sequence identity with SnSAG1. Analysis of these alignments indicates that the sequence of the SnSAG1 cDNA from the UC1 isolate described here is essentially identical to that from the SN3 isolate from which the EST's were prepared.

The gene encoding SAG1 from S. neurona differs from those encoding the corresponding SAG1 of T. gondii and N. caninum, which are encoded without an intron. This difference is additional evidence of the larger phylogenetic distance between Sarcocystis spp. and these other coccidians than that which exists between T. gondii and the Neospora spp. (Mugridge et al., 1999, 2000). The sequence of SnSAG1 indicates that addition of a GPI anchor is likely, since it appears to encode cleavable N-terminal and C-terminal signal sequences and has a single transmembrane spanning region near the C terminus. We do not currently have experimental data that would confirm the presence of a GPI anchor, but this post-translational modification is consistent with the structure of all major surface antigens on T. gondii (Lekutis et al., 2001).

The gene and its protein product, plus the antibodies raised against it, provide tools with which to study the pathogenesis of disease in the horse. The nucleic acids encoding SnSAG1 may be useful in identifying S. neurona merozoites, sporocysts, or sarcocysts, particularly since the mRNA is abundant. The mRNA that encodes SnSAG1 has been amplified both from sarcocysts found in armadillo muscle and sporocysts from an opossum (data not shown) in addition to merozoites in culture. Antibodies in clinical fluids from a horse diagnosed with EPM bind to rSnSAG1 on protein blots, but comparable antibodies from horses that were never exposed to S. neurona do not bind suggesting a role for this recombinant antigen in diagnostics. In vitro growth of S. neurona merozoites in the presence of mAb 1631 does not prevent attachment, invasion, or replication in host cells (data not shown), thus the epitope recognised on SnSAG1 does not appear to be essential for these processes. The Mab 1631 recognises parasites in tissue preparations by immunohistochemistry, and beads coated with mAb 1631 (Pathogenes, Inc.) have been used successfully to recover S. neurona from clinical tissues for in vitro culture (data not shown). Monoclonal antibodies 2A7 and 1631 bound the native 29 kDa antigen and rSnSAG1 best under non-reducing conditions, indicating that the folding of the protein contributes to the conformation of the epitopes recognised by these mAbs. We have demonstrated here that these antibodies bind antigens from two isolates of S. neurona. Additionally, these antibodies bound four other isolates (data not shown); therefore, the epitope(s) recognised is(are) common among S. neurona isolates.

Molecular approaches to the analysis and production of specific antigens of S. neurona will underpin future efforts

Fig. 2. Epitopes on rSnSAG1 recognised by mAb 2A7 are destroyed by thioredoxin reduction. Lane 1, rSnSAG1 (non-reduced); lane 2, rSnSAG1 (reduced by boiling in 5% β-mercaptoethanol); lane 3, rSnSAG1 (reduced by boiling in 10% β-mercaptoethanol).
num is more closely related to Hammondia heydorni than to Toxoplasma gondii. Int. J. Parasitol. 29, 1545–56.


to improve diagnosis and prevent EPM in horses. Antemortem testing of horses from North America for EPM is complicated by the high prevalence (>50%) of serum antibodies to \textit{S. neurona}, and the low prevalence of the clinical disease (~0.2%) (MacKay, 1997; NAHMS, 2001). Diagnosis of EPM is therefore based on detecting antibodies against \textit{S. neurona} in the CSF of horses with neurological deficits. Since SnSAG1 is a prominent merozoite antigen, it is of some interest to assess the utility of rSnSAG1 as a target antigen in an assay for intrathecal, anti-\textit{S. neurona} antibodies in clinically affected horses. Such testing must resolve concerns about cross-reactive antibodies resulting from infections with other \textit{Sarcocystis} spp., antigenic differences among \textit{S. neurona} isolates, and differences in the immune response to infection. Antigenic differences have not been detected among the several isolates examined here, but the nucleic acids encoding SnSAG1 from many additional \textit{S. neurona} isolates need to be examined to discover naturally occurring allelic variants. SnSAG1 is also a logical candidate to evaluate for inclusion in a subunit vaccine, since a vaccine employing a recombinant form of \textit{T. gondii} SAG1 was shown to be effective in a maternal/foetal guinea pig model of infection with that parasite (Haunmont et al., 2000) and protective immunity was also induced in animals using a surface antigen of \textit{N. caninum} expressed on a herpes virus vector (Nishikawa et al., 2000).

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