The SnSAG merozoite surface antigens of *Sarcocystis neurona* are expressed differentially during the bradyzoite and sporozoite life cycle stages

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**A B S T R A C T**

*Sarcocystis neurona* is a two-host coccidian parasite whose complex life cycle progresses through multiple developmental stages differing at morphological and molecular levels. The *S. neurona* merozoite surface is covered by multiple, related glycosylphosphatidylinositol-linked proteins, which are orthologous to the surface antigen (SAG)/SAG1-related sequence (SRS) gene family of *Toxoplasma gondii*. Expression of the SAG/SRS proteins in *T. gondii* and another related parasite *Neospora caninum* is life-cycle stage specific and seems necessary for parasite transmission and persistence of infection. In the present study, the expression of *S. neurona* merozoite surface antigens (SnSAGs) was evaluated in the sporozoite and bradyzoite stages. Western blot analysis was used to compare SnSAG expression in merozoites versus sporozoites, while immunocytochemistry was performed to examine expression of the SnSAGs in merozoites versus bradyzoites. These analyses revealed that SnSAG2, SnSAG3 and SnSAG4 are expressed in sporozoites, while SnSAG5 was appeared to be downregulated in this life cycle stage. In *S. neurona* bradyzoites, it was found that SnSAG2, SnSAG3, SnSAG4 and SnSAG5 were either absent or expression was greatly reduced. As shown for *T. gondii*, stage-specific expression of the SnSAGs may be important for the parasite to progress through its developmental stages and complete its life cycle successfully. Thus, it is possible that the SAG switching mechanism by these parasites could be exploited as a point of intervention. As well, the alterations in surface antigen expression during different life cycle stages may need to be considered when designing prospective approaches for protective vaccination.

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1. Introduction

*Sarcocystis neurona* is a coccidian that is the primary cause of Equine Protozoal Myeloencephalitis (EPM). EPM is the most commonly diagnosed neurological disease of horses causing an estimated annual loss of more than 100 million dollars in the United States (Dubey et al., 2001b). In addition to causing problems in horses, neurologic disease in sea mammals has been attributed to *S. neurona* (Rosonke et al., 1999; Lindsay et al., 2000; Dubey et al., 2001c; Miller et al., 2001; Thomas et al., 2007). The natural life cycle of this parasite alternates between the definitive host, the opossum (Fenger et al., 1995), and small mammal intermediate hosts such as skunks (Cheadle et al., 2001b), raccoons (Dubey et al., 2001d), armadillos (Cheadle et al., 2001a), sea otters (Dubey et al., 2001c), and cats (Dubey et al., 2000). Sporulated oocysts containing infectious sporozoites are a product of sexual reproduction, which takes place in...
the intestine of the opossum definitive host. This environmentally resistant cyst stage is shed in the opossum feces and serves as the source of infection for intermediate hosts. Asexual reproduction of *S. neurona* in the intermediate hosts is characterized by two developmental stages called merozoites and bradyzoites. Merozoites propagate rapidly in a variety of cell types through a process called endopolygeny, while bradyzoites are a much slower-growing stage that form sarcocysts, most commonly in muscle tissue (Dubey et al., 2001a; Speer and Dubey, 2001).

The cell surface of *S. neurona* merozoites is covered with an array of paralogous glycosylphosphatidylinositol (GPI)-anchored surface antigens called SnSAGs (Howe et al., 2005). These surface proteins were identified based on their homology to the gene family of TgSAGs and SAG1-related sequences (SRSs) in the related parasite *Toxoplasma gondii* (Lekutis et al., 2001). A total of six SnSAGs have been described in *S. neurona*, with only a subset of these SnSAGs expressed by individual parasite strains (Howe et al., 2005; Crowdsus et al., 2008; Wendte et al., 2010). In *T. gondii*, a search of the parasite genome has revealed a superfamily of 161 SAG/SRS proteins (Jung et al., 2004).

The functional role of these surface antigens has not been fully defined, but there is evidence to suggest that the SAG proteins are involved in host cell invasion, immune modulation and/or virulence attenuation (Mingo and Kasper, 1994; Grimwood and Smith, 1996; Kim and Boothroyd, 2005; Saeij et al., 2008).

Stage-specific expression of the SAG/SRS proteins has been observed in both *T. gondii* and *Neospora caninum* (Tomavo et al., 1991; Odberg-Ferragut et al., 1996; Fuchs et al., 1998; Knoll and Boothroyd, 1998; Scharer et al., 1999; Lekutis et al., 2000; Radke et al., 2004; Fernandez-Garcia et al., 2006; Risco-Castillo et al., 2007; Saeij et al., 2008). It has been proposed that the tachyzoite-specific SAGs are involved in regulation of virulence and elicitation of immune response to give rise to an acute infection, while the bradyzoite-specific SAG molecules may be important for immune evasion and persistence of a chronic infection (Kim and Boothroyd, 2005; Saeij et al., 2008). Less is known of SAG/SRS gene family members expressed during the sporozoite stage. In the current study, we have examined the *S. neurona* SnSAGs that have been identified in merozoites to determine whether these proteins are expressed constitutively or in a stage specific manner. Our analyses demonstrated that these merozoite surface antigens are differentially expressed in the bradyzoite and sporozoite stages of *S. neurona*, consistent with the findings for other coccidians.

### 2. Materials and methods

#### 2.1. Parasites

*S. neurona* strain SN138 (Lindsay et al., 2004), which is an independent culture derived from the SN-37R strain (Sofaly et al., 2002), was maintained in bovine turbinate cells, and extracellular merozoites were harvested as described previously (Howe et al., 2005). The SN-37R strain and SN138 sub-strain lack the SnSAG1 gene and instead express SnSAG5 (Crowdsus et al., 2008; Howe et al., 2008). Strain SN-37R sporozoites were produced previously in laboratory-reared opossums (Sofaly et al., 2002). Bradyzoites were recovered from muscles of a raccoon euthanized 3 months after oral inoculation with strain SN-37R sporocysts (Sofaly et al., 2002). Muscle tissue was ground briefly in a blender, and pre-warmed acid-pepsin solution was added and incubated at 37 °C for 10 min with shaking, as described previously (Dubey et al., 1989). The muscle homogenate was centrifuged, washed with saline solution, centrifuged again, and the supernatant and muscle layer were discarded.

#### 2.2. Western blot analysis

Merozoites and sporozoites were subjected to lysis in SDS sample buffer supplemented with 2-mercaptoethanol and a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma, St. Louis, MO, USA). For sporozoite protein, 1.5 ml of sporocysts (Sofaly et al., 2002) was pelleted at 5000 rpm for 15 min. The pellet was resuspended in 500 μl of sodium dodecyl sulfate (SDS) sample buffer, and homogenized for 2 min using a Heidolph homogenizer (Sigma-Aldrich). Antigen equaling 5 × 10⁴ culture-derived merozoites per lane was separated on 12% polyacrylamide gels (Laemmli, 1970). The approximate equal amount of sporozoite antigen was determined empirically based on the amount of parasite actin detected by Western blot in the two life cycle stages. The separated proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris–glycine buffer (pH 8.3). The membranes were blocked for 30 min with phosphate-buffered saline (PBS) containing 5% non-fat dry milk (NFDM), 5% normal goat serum (NGS), and 0.05% Tween 20, followed by primary antibody incubation for 1 h in PBS containing 0.1% NGS, 0.1% NFDM, and 0.05% Triton X-114. Primary antibodies were rabbit monospecific polyclonal antisera that had been produced previously against each recombinant SnSAG (Howe et al., 2005; Crowdsus et al., 2008). After multiple washes, the membranes were incubated with peroxidase-conjugated, goat anti-rabbit immunoglobulin G secondary antibody (Jackson Immunoresearch Labs, Inc., West Grove, PA, USA) for 1 h. The membranes were then washed and incubated with Supersignal substrate (Pierce, Rockford, IL, USA) for chemiluminescence detection, and visualized with a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA).

#### 2.3. Immunocytochemistry

For examination of merozoites, culture-derived parasites were harvested and diluted in PBS. A drop of the parasite suspension was smeared and air dried on electrostatically treated Superfrost Plus slides (Fisher Scientific). The slides were fixed for 10 min in cold acetone kept at −20 °C, followed by washing in deionized H₂O (dH₂O). For examination of bradyzoites, the organisms recovered from raccoon muscle (described above) were smeared on IFA or saline slides and fixed with cold methanol. The slides were washed in dH₂O for 1 min and then subjected to
heat-induced antigen unmasking by microwave irradiation in 10 mM sodium citrate buffer, pH 6.0. After allowing cooling at room temperature, the slides were washed with PBS–Tween 20 (PBST). Immunocytochemical labeling of parasites was performed using the ImmunoCruz staining system (rabbit: Santa Cruz Biotechnology Inc., Santa Cruz, CA) as per the manufacturer’s protocol, with slight modifications. Briefly, endogenous peroxidase was quenched by incubating with peroxidase blocking solution followed by washing with PBST. Non-specific antigen sites were blocked in rabbit serum at room temperature (RT) for 1 h. Primary antibody incubation was carried out for 1 h at RT followed by washing with PBST. Primary antibodies were monospecific polyclonal antiserum against each SnSAG, as described above. This was followed by incubation with biotinylated secondary antibody for 30 min at RT, followed by washing with PBST. An incubation of 30 min was performed at RT with horseradish peroxidase (HRP)–streptavidin complex, followed by washing with PBST and incubation with HRP substrate for 5 min at RT. Slides were counterstained for 1 min with hematoxylin, dehydrated, and covered with a glass coverslip using 1–2 drops of permanent mounting medium (Surgipath Medical Ind., Inc., Richmond, IL). Normal rabbit serum containing IgG (provided with the kit) served as a negative control, while polyclonal antibody raised against the whole S. neurona merozoite antigen was used as a positive control.

The slides were examined using a Nikon Microscope Labophot-2 (Nikon Instruments Inc., Melville, NY), and images were visualized and captured with a Nikon DS-Fi1 camera head and DS-L2 camera control unit. Images were enhanced by adjusting the brightness and sharpness in Adobe Photoshop Elements 9.

3. Results

3.1. SnSAG expression in sporozoites

In order to identify the SnSAG surface antigens expressed by S. neurona sporozoites, Western blot analysis was performed using polyclonal antiserum raised against each of the merozoite SnSAGs. These analyses revealed SnSAG2, SnSAG3, and SnSAG4 in the sporozoite stage. Actin served as a control for protein loading. Membranes were incubated with rabbit polyclonal antiserum against parasite actin (1:5000), SnSAG2 (1:10,000), SnSAG3 (1:10,000), SnSAG4 (1:5000) and SnSAG5 (1:5000). M = merozoite; S = sporozoite.

3.2. SnSAG expression in bradyzoites

Since a relatively small number of S. neurona bradyzoites were available for analysis, it was not possible to examine this life cycle stage by Western blot. To assess SnSAG expression in the bradyzoite stage, immunocytochemistry was performed on parasites that had been isolated from sarcocysts and applied to slides. As shown in Fig. 2, culture-derived merozoites and the cyst-derived bradyzoites exhibited staining with the positive control antiserum against S. neurona whole-merozoite antigen, while no staining of these parasite stages was observed with the negative control serum. Polyclonal antiserum against SnSAG2, SnSAG3, and SnSAG5 intensely labeled the merozoites, while no labeling was observed for the bradyzoite stage using these antiserum (Fig. 2). Merozoites labeled inconsistently with the anti-SnSAG4 antisera; which was likely due to the acetone fixation since merozoites fixed with glutaraldehyde were efficiently labeled (data not shown), but no labeling of bradyzoites was observed with the anti-SnSAG4 antibodies. Collectively, the immunocytochemistry results suggest that the SnSAG2, SnSAG3, SnSAG4 and SnSAG5 merozoite surface antigens are absent or much less abundant in the bradyzoite stage of S. neurona. As mentioned for the Western blot analyses,
Fig. 2. Immunocytochemical labeling of Sarcocystis neurona merozoites and bradyzoites demonstrated that the SnSAG merozoite surface proteins are downregulated in the bradyzoite stage. Both merozoites and bradyzoites were labeled with the positive control serum, while labeling with α-SnSAG2, α-SnSAG3, α-SnSAG4 and α-SnSAG5 was only apparent in merozoites. Rabbit primary antisera were against whole S. neurona merozoite lysate (positive control), SnSAG2, SnSAG3, SnSAG4, and SnSAG5 (1:1500 dilutions). Normal rabbit serum was used as the negative control.

SnSAG1 was not examined in bradyzoites since this paralogue is not present in the parasite strain used for this study.

4. Discussion

Two-host coccidian parasites, which include S. neurona, have complex life cycles that require conversion between distinctive developmental stages. Associated with these developmental switches are morphological, metabolic, and molecular alterations that differentiate the life cycle stages and permit parasite transmission and survival in a new environment (Tomavo, 2001; Lyons et al., 2002; Marugan-Hernandez et al., 2010). Documentation of these molecular changes can provide a better understanding of the stage conversion process as well as some insights into the function of individual proteins or protein families. The results of the present study demonstrated that the SnSAG merozoite
surface antigens of *S. neurona* are expressed in a stage-specific manner. Specifically, the SnSAG5 major surface antigen was mostly absent from sporozoites in Western blot analysis, while all four of the SnSAGs described in the SN37-R strain (aka SN138) were undetectable on bradyzoites by immunocytochemistry. These findings imply that alteration of the parasite surface is important during *S. neurona*'s developmental progression through its major life cycle stages.

All three life cycle stages of *S. neurona* have been produced for only a single parasite strain (SN37-R), so it is not possible currently to confirm the present findings in other *S. neurona* strains such as those that possess SnSAG1 rather than SnSAG5 (Howe et al., 2008). However, the stage-specific expression of the SnSAGs described in the present study is consistent with multiple previous findings for related coccidian parasites, so it is reasonable to speculate that the phenomenon occurs in strains of *S. neurona* other than SN37-R. In *T. gondii*, the tachyzoite surface displays a mixture of SAG/SRS molecules, including TgsAG1, SAG2A, SAG3, SRS1, SRS2, SRS3 as well as several other less highly expressed SRSs (Manger et al., 1998). In contrast, an alternative array of SAGs/SRSs such as SAG2C and D, SRS9, SAG4 and BSR4 appear to decorate the *T. gondii* bradyzoite surface (Odberg-Ferragut et al., 1996; Knoll and Boothroyd, 1998; Lekutis et al., 2000). Likewise, NcSAG1 and NcSRS2 have been found to be tachyzoite specific in *N. caninum* (Fuchs et al., 1998; Scharaschkin et al., 1999), while the proteins NcSAG4 and NcBRS4 are specifically expressed during the *N. caninum* bradyzoite stage (Fernandez-Garcia et al., 2006; Risco-Castillo et al., 2007). Sporozoites of *T. gondii* appear to lack the tachyzoite surface proteins TgsAG1 and TgsAG2A, but instead express SporoSAG and a still-undefined surface protein of approximately 67 kDa (Kasper et al., 1984; Boothroyd et al., 1998; Radke et al., 2004).

There is significant evidence that the SAG/SRS gene families in coccidian parasites function in both host cell adhesion/invasion and in immune modulation and parasite persistence. Multiple studies utilizing monoclonal antibodies or SAG-deficient mutants of *T. gondii* have implicated a role for various members of this gene family in the process of adhering to and invading host cells, although the results of these studies suggested that each SAG/SRS parologue might function differently in the process (Grimwood and Smith, 1992; Mineo and Kasper, 1994; Grimwood and Smith, 1996; Dzierzinski et al., 2000). In addition, it is apparent that expression of the bradyzoite-specific SAG/SRS proteins is important for the parasite’s ability to establish and maintain latent cysts in host tissues (Kim and Boothroyd, 2005; Kim et al., 2007; Saeij et al., 2008). These studies collectively suggest that *T. gondii* persistence in the intermediate host is aided by both antigen switching to evade immune killing and by specific attributes of the bradyzoite-specific SAG/SRS gene family members (i.e., SRS9 and SAG2C/DXY). It has been further speculated that SAG/SRS switching during stage transition serves to prepare the parasites for invasion of new cell types, which is an appealing concept given the vast array of host animals and tissues that *T. gondii* might encounter during its complex life cycle. It is interesting to note that *T. gondii* has an extensive repertoire of SAG/SRS paralogues that can be expressed during the life cycle (Jung et al., 2004), consistent with the expansive host range of this parasite, while examination of the draft genome for *S. neurona* revealed a much more limited number of SAG/SRS loci (A. Gautam and D. Howe, unpublished data). It will be important to determine whether any of these uncharacterized SnSAG genes are specifically expressed during the bradyzoite or sporozoite stages of *S. neurona*. As well, it could be highly informative to assess whether individual SAG proteins exhibit specific tissue or cell tropisms.

In conclusion, it appears that surface antigen switching during developmental stage conversion is conserved in the two-host coccidia and is presumably important for successful completion of the parasite life cycle. Therefore, it is conceivable that stage-specific SAG/SRS expression and surface antigen switching could be exploited for intervention of infection. In the case of *S. neurona* infection in horses, it remains uncertain whether the parasite normally converts to the bradyzoite stage, so identification of bradyzoite-specific SnSAG paralogues may shed light on this question. As well, it is clear that sporozoites of *S. neurona* are the only stage capable of infecting horses. Thus, it will be likely important to consider the array of SnSAGs expressed during the sporozoite stage if developing a vaccine that is intended to block parasite invasion in the equine gut.

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