Invited review

Surface antigens of *Toxoplasma gondii*: variations on a theme

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Abstract

*Toxoplasma gondii* is an obligate intracellular protozoan parasite with an exceptionally broad host range. Recently, it has become apparent that the number of surface antigens (SAGs) it expresses may rival the number of genera it can infect. Most of these antigens belong to the developmentally regulated and distantly related SAG1 or SAG2 families. The genes encoding the surface antigens are distributed throughout the *T. gondii* genome, with remarkably little polymorphism observed at each locus. Results from a number of studies have suggested that the surface antigens play an important role in the biology of the parasite. For example, SAG3 null mutants generated by targeted disruption provide convincing evidence that this surface antigen at least functions during parasite attachment. Analyses of a SAG1 knockout in rodents, however, indicate that this surface antigen may play a crucial role in immune modulation or virulence attenuation. The current understanding of the SAG1 and SAG2 families will be discussed here. © 2001 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

*Toxoplasma gondii* is a medically important pathogen capable of causing death in utero and in immunocompromised individuals (Guerina, 1994; Luft and Remington, 1992). As such, a great deal more has been learned about this parasite than about other apicomplexans threatening only wild-life or domestic animals. *Toxoplasma gondii* appears able to infect virtually any tissue of all mammalian and avian species examined (Miller et al., 1972). This is in contrast to the apparently more restricted host range of other Sarcocystidae family members, *Neospora* sp., *Sarcocystis* sp., and *Hammondia* sp. (Dubey, 1993).

The life-cycle of this obligate intracellular parasite includes an asexual phase occurring in all hosts and a sexual phase occurring only in the gut of its definitive host, the cat (Frenkel et al., 1970; Hutchison et al., 1970; Sheffield and Melton, 1970). There are four functionally distinct forms of *T. gondii*: tachyzoite, bradyzoite, merozoite and sporozoite. Tachyzoites rapidly proliferate and quickly disseminate within the host indiscriminately infecting many cell types in the process. Bradyzoites slowly grow within cysts located in brain and muscle tissue and can infect new hosts upon ingestion. Merozoites result from asexual division in feline enterocytes, which is necessary to increase parasite density prior to sexual development. Sporozoites are the product of the sexual phase and like bradyzoites can establish an infection upon ingestion. Thus, there is considerable variation in cell tropism among the four infectious forms of *T. gondii*.

The surface of *T. gondii* tachyzoites and bradyzoites is covered with glycosylphosphatidylinositol (GPI)-anchored antigens (Nagel and Boothroyd, 1989; Tomavo et al., 1989), most of which are members of the surface antigen 1 (SAG1) or SAG2 families (Lekutis et al., 2000; Manger et al., 1998; Boothroyd et al., 1998). These molecules appear to play a role in host cell invasion, immune modulation and/or virulence attenuation, although they may also provide protection needed by the parasite to survive in the environment. For instance, a different covering may be required by tachyzoites in the avian lung than is required by bradyzoites in the feline gastrointestinal tract. Here we will describe the current understanding of the SAG families and discuss their possible function.

2. Genes

Through the use of mAbs and radio-iodination, multiple
T. gondii SAGs were first identified biochemically. The genes which encode three of these, P30 (SAG1), P22 (SAG2A) and P43 (SAG3), were subsequently identified using antigen-specific mAbs to screen expression libraries (Table 1) (Burg et al., 1988; Cesbron-Delauw et al., 1994; Prince et al., 1990). Shortly thereafter, limited amino acid sequence was obtained from affinity-purified P18, permitting the corresponding gene (SAG4A) to be cloned by traditional molecular methods (Odberg-Ferragut et al., 1996). Two additional SAG genes were cloned by genome walking (SRS1) and gene trapping (BSR4, P36), respectively (Hehl et al., 1997; Knoll and Boothroyd, 1998). Analysis of the T. gondii expressed sequence tag (EST) database next revealed the existence of another SAG4-like gene (SAG4B), as well as multiple SAG1-related sequences (SRS2, SRS3 and SRS4) (Ajikoya et al., 1998; Manger et al., 1998). The SAG1 family was thus defined as those genes encoding GPI-anchored proteins that maintain the 12 conserved cysteine residues of SAG1 and which have an overall identity of approximately 30%. At that time, the SAG1 family consisted of SAG1, SAG3, SRS1-SRS4 and BSR4, while SAG2A and SAG4A-B were considered to be unrelated to this family.

Several investigators have now described additional SAG1 family members. Spano and colleagues have cloned three tandemly arrayed SAG1-like genes (SAG5A, SAG5B and SAG5C) (pers. commun.). SAG5B and SAG5C are strikingly similar to each other throughout their open reading frames, whereas the amino-terminal half of SAG5A is notably dissimilar. By genome walking, we have recently cloned two SAG1-related sequences adjacent to BSR4 (SRS5 and SRS6). SRS5 is located ~1.1 kb upstream of BSR4, and SRS6 is found ~1.6 kb upstream of SRS5. Four stop codons and a frameshift are found within the most likely reading frame of SRS5 indicating that it is a pseudogene, while SRS6 may be functional. We have also cloned two additional SAG1-related sequences (SRS7 and SRS8) by PCR using primers designed from a lone EST sequence. The predicted products of SRS5 (stop codons and frameshift notwithstanding) and SRS6, as well as the newly described SRS7 and SRS8, are most similar to that of SRS4 and BSR4 (Fig. 1).

A second family of SAG genes defined by homology to SAG2 (SAG2B, SAG2C and SAG2D) have been identified in a similar cyber-assisted manner (Lekutis et al., 2000). Sequence analysis has revealed that the SAG2 family is in fact distantly related to the SAG1 family (Fig. 1). In an independent effort, Mattsson and colleagues have cloned a SAG2-related gene termed SAG2E (pers. commun.). Members of the SAG2 family share only a subset of their cysteine residues and tend to be less similar to each other than are members of the SAG1 family (~20% identity within the SAG2 family versus ~30% identity within the SAG1 family). Interestingly, significant differences exist in the sizes of the open reading frames of the SAG2 family. Both SAG2B and its prototype SAG2A have much shorter open reading frames (~200 amino acids) than do the other three SAG2-related sequences and the immediate members of the SAG1 family (~300 amino acids). That said, the predicted products of SAG2C and SAG2D are remarkably alike (~80%), particularly at their carboxyl-termini (second

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* #aa, number of amino acids.
* NA, not known.
* NA, not applicable.
Fig. 1. Unrooted phylogenetic tree of the SAG1 superfamily constructed from a ClustalW amino acid alignment and drawn using the WebPhylip package (Felsenstein, 1989; Thompson et al., 1994). An open reading frame for SRS5 was constructed by ignoring the three premature stop codons and correcting the frameshift to preserve the canonical SAG1 family structure. Asterisks indicate that for SRS7 and SRS8, only partial open reading frames were available for this analysis. The multiple sequence alignment was constructed with ClustalW (1.81) from the European Bioinformatics Institute web site specifying phylip as the output format and aligned as the output order. The aligned sequences were then subjected to distance computation using the PAM matrix with WebPhylip (1.2) from the National University of Singapore web site. The neighbour-joining method was then used to construct the phylogeny, which was subsequently visualised as a postscript file with branch lengths of 0.1 and an enthusiasm constant of 0.001. ClustalW. http://www2.ebi.ac.uk/clustalw/; WebPhylip. http://sdmc.krdl.org.sg:8080/~lxzhang/phylip/

exon). Additionally, SAG2C and SAG2D are unique in that these genes possess a single small intron, while all other known T. gondii SAGs are expressed from unspliced transcripts.

3. Orthologues

The closely related coccidian Neospora caninum causes canine paralysis, as well as bovine abortion (Dubey, 1993). Two immunodominant SAGs are expressed by N. caninum tachyzoites. These antigens were originally termed Ncp36 and Ncp43 based upon their mobility through reducing SDS-PAGE (Hemphill et al., 1997a,b; Hemphill and Gottstein, 1996), or Ncp29 and Ncp35 when analysed under non-reducing conditions (Howe et al., 1998). The corresponding genes were cloned by Hemphill and colleagues through screening a N. caninum expression library with affinity-purified antiserum (Hemphill et al., 1997a,b; Sonda et al., 1998). Howe et al. (1998) confirmed these results through determination of internal amino acid sequence of Ncp29 and Ncp35, immunoprecipitated by mAbs 6C11 and 5H5, respectively. Comparison of the N. caninum gene products with those of T. gondii revealed that Ncp36/29 is most closely related to SAG1, while Ncp43/35 is most closely related to SRS2 (Fig. 1). Thus, the gene encoding the smaller N. caninum SAG has been named NcSAG1, whereas the larger SAG gene has been named NcSRS2 (Howe and Sibley, 1999). More recently, Marsh et al. (1999) have cloned the SAG1 and SRS2 orthologues of Neospora hughesi, which are 94 and 91% identical at the amino acid level to their N. caninum counterparts.

Sarcocystis is another genus whose constituent species are morphologically similar to T. gondii. Several SAG ESTs (expressed sequence tags) were identified from the newly initiated Sarcocystis neurona effort, although a complete contig could not be assembled. Eschenbacher et al. used the mAb SM-17 to clone the cyst SmS31 gene from a Sarcocystis muris expression library (Eschenbacher et al., 1992; Menceke et al., 1991; Tenter et al., 1991). Interestingly, the carboxyl-terminus of SmS31 (SmSAG) was found to resemble SAG2A, particularly in the number and spacing of cysteine residues (Cesbron-Delaup, 1995). The similarity of SmS31 to the longer SAG2C, SAG2D and SAG2E is even more striking (Fig. 1). In contrast, the amino-terminal half of SmS31 more closely resembles serine and threonine-rich mucins, the significance of which is unclear.

4. Expression

Although 21 Toxoplasma SAG genes have been cloned, much remains to be learned in regard to their pattern of expression (Table 1). Only SRS5 appears to be a pseudogene, while expression of SRS4, SRS6, SRS7, SRS8, SAG2E, and SAG4B has yet to be demonstrated. Through the use of antiserum raised against maltose binding protein (MBP)-SAG2B-D fusions, SAG2B was detected as a ~23 kDa band in non-reduced RH lysates, while SAG2C and/or SAG2D expression was observed by IFA (Lekutis et al., 2000). Unfortunately, the MBP-SAG2C/D-reactive antiserum did not detect a protein by Western blot, hence the molecular weight of SAG2C and SAG2D remain undefined. The SAG5 genes also appear to be functional, although at this time only SAG5B/C expression has been observed (Spano, pers. commun.).

The new SAG genes are good candidates for the uncloned T. gondii proteins defined previously by mAb P35, P34, P23 and P21 (Couvreur et al., 1988; Tomavo et al., 1991). In an effort to correlate new genes with old proteins, serial immunoprecipitations and Western blots were done. In this way, SAG2B was found to be distinct from P23 defined by mAb 2E12 (Lekutis et al., 2000). In contrast, P35, defined by Couvreur et al. (1988), appears to be encoded by SRS3. As shown in Fig. 2, P35 immunoprecipitated by mAb 3F12 was detected by antiserum raised against an MBP-SRS3 fusion protein. Unfortunately, the converse experiment could not be done, as the SRS3-reactive antiserum does not immunoprecipitate SRS3, although it does detect this protein in Western blots. Thus, it is also
formally possible that SRS3 and P35 are distinct entities that share (a) common epitope(s). Interestingly, P35 defined by Handman et al. is not recognised by the SRS3-reactive antiserum or mAb 3F12, indicating that P35 identified in the Dubremetz laboratory is distinct from that identified by Remington and colleagues (data not shown) (Handman, 1980, #56). Additional SAG genes must exist since three

\[ T. gondii \text{ surface proteins are not known to be encoded by any of the genes so far identified (P34, P23 and P21). Whether these will prove to be homologues of SAG1 or SAG2, or members of a completely new family remains to be determined.} \]

5. Differentiation

With the exception of SAG1, a complete expression profile is lacking for many of the SAG genes in the four forms of \( T. gondii \) (tachyzoite, bradyzoite, merozoite and sporozoite). Even so, there appears to be significant developmental regulation of their expression. Not surprisingly, there is a direct correlation between the ease with which a stage can be studied and the depth of our knowledge of that stage. The highly infectious sporozoite stage is the result of sexual development and oocyst formation in the cat intestine and is only found in the faeces of the definitive host (felines). As such, few sporozoite antigens have been described biochemically or molecularly. Sporozoites express two major immunogenic SAGs of ∼67 and ∼25 kDa (uncloned) in the absence of the immunodominant tachyzoite antigens SAG1 and SAG2 (Kasper et al., 1984; Kasper and Ware, 1985).

There have been no molecular studies conducted on the merozoite stage, although the expression of several SAGs has been examined by immunocytochemistry. Interestingly, antiserum raised against a MBP-BSR4 fusion protein reacts with the surface of merozoites in the cat gut, as well as bradyzoites but not tachyzoites in the mouse brain. This suggests that both enteric and exoenteric forms of \( T. gondii \) express BSR4 or a BSR4-like molecule (Fig. 3). It has also been observed that merozoites fail to express SAG1, SAG2C/D and SRS2. In these studies, similarly fixed and processed tissues containing various developmental stages were stained in parallel to reduce the possibility of artefacts.

Early bradyzoite studies relied upon stage-specific antiserum and radio-iodination to describe the surface of this stage (Kasper, 1989; Lunde and Jacobs, 1983). Subsequently, a panel of mAbs was used to define four bradyzoite-specific molecules (P36, P34, P21 and P18) (Tomavo et al., 1991). The development of tools such as γ-interferon, elevated temperature and alkaline pH, which can be used to induce differentiation in vitro, has permitted the bradyzoite stage to be studied in greater detail (Bohne et al., 1993; Soete et al., 1994). For instance, tissue culture-derived bradyzoite material was used to clone the genes encoding both SAG4 (P18) and BSR4 (P36) (Knoll and Boothroyd, 1998; Odberg-Ferragut et al., 1996). More recently, Spano and colleagues have determined that SAG5A is developmentally regulated and is transcribed exclusively by bradyzoites (pers. commun.). Chronically infected rodents, however, remain a useful resource for the study of mature bradyzoite markers (i.e. P21), as their expression may not be adequately stimulated under typical culture conditions or...
when using laboratory-adapted strains (Soete et al., 1993, 1994). In fact, SAG2C and/or SAG2D expression was not detected in pH-induced bradyzoites, although their expression by in vivo-derived bradyzoites was readily observed (Lekutis et al., 2000).

Five major antigens on the tachyzoite surface were initially defined by mAb (P43, P35, P30, P23 and P22) (Couvreur et al., 1988). While two of these, P43 (SAG3) and P23, are also expressed by bradyzoites (Tomavo et al., 1991), the others and several of the more recently identified SAGs are developmentally regulated. For instance, the SAG2 family members SAG2A and SAG2B have so far been detected only on tachyzoites, while SAG2C and/or SAG2D have been detected only on mature bradyzoites (Lekutis et al., 2000). Additionally, the SAG1 family members SAG5B and SAG5C, but not SAG5A, are expressed by tachyzoites (Spano, pers. commun.). The modest conservation in primary amino acid sequence and the predicted similarity in higher order structure suggest that significant cross-reactivity may occur when polyclonal reagents are used to examine SAG expression. For this reason, the development of truly antigen-specific mAbs such as those raised against unique linear peptide epitopes is worthwhile. To this end, an SRS2 peptide-specific mAb (CL23) raised against GPPYR-YEPEKFT was used to convincingly demonstrate that SRS2 expression is tachyzoite-specific (Fig. 4).

6. Variation

In addition to developmentally regulated differences in SAG expression, there is measurable allelic variation between the three prototypic strains of T. gondii (Howe and Sibley, 1995). Interestingly, just two alleles were identified at the SAG1 and SAG2A loci when Type I, II and III strains were compared (Bulow and Boothroyd, 1991; Parmley et al., 1994). In fact, most SAGs are dimorphic when the three strain types as well as some rare ‘recombinant’ isolates are compared, with typically ~1–5% polymorphism observed over 0.5–2.0 kb sequenced (Grigg, Bonnefoy, Hehl, and Boothroyd, unpublished data). SAG dimorphism and chromosomal localisation are windows through which the population biology of T. gondii can be observed. This growing collection of data indicates that current T. gondii isolates may be products of sexual recombination between two unidentified founder strains (Grigg et al., unpublished data).
7. Function

Through the use of mAbs, it has become apparent that the GPI-linked SAGs of *T. gondii* function at an early stage during the process of invasion. A subset of mAbs directed against P30 (SAG1) reproducibly inhibit tachyzoite attachment to cultured host cells (Grimwood and Smith, 1992; Mineo et al., 1993). In contrast, incubation of parasites with either of two P22 (SAG2A)-specific mAbs (T4.3G11 and T4.1F5) enhanced tachyzoite attachment (Grimwood and Smith, 1996). Thus, it appears that the SAGs are not functionally identical.

The importance of SAG1 and SAG2 during invasion was simultaneously validated and diminished by the isolation of viable SAG mutants. These mutants were generated using chemical mutagenesis followed by negative selection using P22 (SAG2A)- or P30 (SAG1)-reactive mAbs in the presence of human complement (Kasper, 1987; Kasper et al., 1982). While these mutants exhibited minor defects in attachment and invasion, the fact that it was possible to isolate P22- and P30-negative mutants indicated that, while these antigens may play a role in invasion, individually they are dispensable. Alternatively, since Kasper’s P22- and P30-negative mutants were generated with relatively high doses of ethynitrosourea and thus possess multiple mutations outside the genes of interest, compensatory changes in the genome may have occurred.

With the advent of reverse genetics in *T. gondii*, the more recent SAG mutants possess precisely defined lesions. Tomavo and colleagues have reported the generation of a SAG3 null mutant by replacement of the wild-type gene with the selectable marker chloramphenicol acetyltransferase (Dzierszinski et al., 2000). Interestingly, SAG3-negative parasites exhibit a two-fold reduction in their ability to adhere to host cells in vitro when compared with both SAG3-complemented and parental RH strains. Even more strikingly, SAG3 null tachyzoites were markedly less virulent than the wild-type or SAG3-complemented strains when administered to BALB/c mice intraperitoneally. The decrease in virulence exhibited by the SAG3 null strain may be due to their diminished capacity to invade host cells, although it is possible that the observed attenuation may be due to a decrease in immune pathology or a reduction in an as yet undescribed function.

More recently, we have engineered a SAG1 null mutant by homologous recombination using the hypoxanthine-xanthine-guanine phosphoribosyltransferase marker (unpublished data). Surprisingly, the SAG1 null clones exhibited a

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**Fig. 4. Immunolocalisation of SRS2 in various developmental stages of *T. gondii*.** In each case SRS2 has been labelled with Texas Red and the second antibody with fluorescein isothiocyanate, while 4',6-diamidino-2-phenylindole has been used to stain the host and parasite nuclei. Bars represent 1 μm. (a) Section of mouse brain at day 15 post-infection showing a group of tachyzoites in which SRS2 (red) co-localises with SAG1 (green) on the surface of the parasites to give a yellow staining pattern. (b) Section of mouse brain at day 28 post-infection in which a cyst (Cy) is unstained with SRS2 and SAG1 but adjacent tachyzoites (T) exhibit surface labelling with SRS2 (red) in the presence of minimal BAG1 staining. (c) Section of mouse brain at day 15 post-infection showing a lesion in which stage conversion is occurring. An early cyst (Cy) with three bradyzoites is positive for BAG1 (green) and negative for SRS2 (red), while adjacent tachyzoites (T) exhibit surface labelling with SRS2 (red) in the presence of minimal BAG1 staining. (d) Similar section to that in (b) showing a cyst strongly labelled with BAG1 (green) but negative for SRS2. (e) Section of rat small intestine illustrating three mature schizonts (S) in which the merozoites are unlabelled with SRS2, but the parasitophorous vacuole (PV) and dense granules within the merozoites are positive for NTPase (green).
time-dependent, two-fold increase in their ability to invade host cells over SAG1-complemented and parental RH controls. Despite this in vitro growth advantage, the SAG1 null parasites were less virulent than controls when administered to outbred Swiss Webster mice intraperitoneally, as determined by a delay in time to death during acute infection. This was in no way due to an inability of this mutant strain to colonise animals, as parasite burden was significantly greater than observed for wild-type or SAG1-complemented control infections. Intra-intestinal infection (injection of parasites directly into the intestine) of susceptible C57BL/6 (B6) mice identified the probable mechanism behind acute virulence of RH parasites (Kasper, pers. commun.). B6 mice readily develop inflammatory bowel disease (IBD) upon intra-intestinal RH infection characterised by gross gut pathology, a massive inflammatory infiltrate and high levels of pro-inflammatory chemokines and TNFα. Infection with SAG1 null parasites resulted in less severe IBD than elicited by wild-type infections and prolonged survival despite 10-fold higher parasitaemia (Kasper, pers. commun.). These results establish that SAG1 potently attracts the immune response and is thereby responsible for the development of the immunopathology accompanying infection of mice with the virulent RH strain. The analysis of additional SAG null mutants, and double/triple knockouts should clarify the role of these molecules in attachment and immune pathology.

8. Conclusions

Apparentl T. gondii possesses an extensive GPI-linked wardrobe (21 and counting). It is tempting to speculate that expression of multiple SAGs may be the mechanism allowing T. gondii to have such a broad tissue and host range. For example, T. gondii may use distinct SAGs to adhere to host cells of distinct species or tissues. If this scenario is true, then it should follow that those Coccidia with narrower host and tissue ranges will possess fewer SAGs.

Alternatively, the SAGs’ major function may be as immune modulators or virulence factors, since the survival of the parasite is ultimately dependent upon the survival of the host. Timely eradication of tachyzoites during acute infection permits bradyzoite development and a life-long chronic infection. Thus, it could be argued that the high level of expression of SAG1, which is limited to the tachyzoite, could assist the host immune response in tachyzoite elimination. Further analysis of the immune response and pathology elicited by infection with SAG null and SAG transgenic parasites should sheds some light on this subject.

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