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ScienceDirect
International Journal for Parasitology 37 (2007) 877–885
www.elsevier.com/locate/ijpara

The BSR4 protein is up-regulated in *Toxoplasma gondii* bradyzoites, however the dominant surface antigen recognised by the P36 monoclonal antibody is SRS9

Tam T. Van [a], Seon-Kyeong Kim [b], Manel Camps [c], John C. Boothroyd [b], Laura J. Knoll [a,*]

[a] *Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 1300 University Avenue, Madison, WI 53706, USA*
[b] *Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA*
[c] *Department of Pathology, University of Washington, HSB K-056, Seattle, WA 91195-7705, USA*

Received 4 December 2006; received in revised form 30 January 2007; accepted 5 February 2007

Abstract

The protozoan parasite, *Toxoplasma gondii*, interconverts between fast-growing tachyzoites and slow-growing bradyzoites within intermediate hosts. The surface of *T. gondii* is covered by the SAG1-related sequence (SRS) superfamily of glycosyl phosphatidyl inositol-anchored proteins, many of which are stage-specific. Previous transient transfection of *BSR4*, a member of the SRS superfamily, showed reactivity with the bradyzoite-specific P36 mAb by immunofluorescene assay. *BSR4* mRNA levels were equally abundant in tachyzoites and bradyzoites, suggesting post-transcriptional regulation of the protein. In this study, we show that BSR4 protein is present in both tachyzoites and bradyzoites, but up-regulated in bradyzoites. However, stable expression of *BSR4* in two BSR4-negative *T. gondii* strains shows minimal reactivity to the P36 mAb by Western immunoblotting, even though the BSR4 protein is abundant. We discovered that the SRS9 protein, a bradyzoite-specific member of the SRS superfamily and encoded immediately downstream of *BSR4*, was also ablated in the BSR4-negative strains, suggesting that SRS9 is the surface antigen recognised by the P36 mAb. Stable expression of *SRS9* in the BSR4 mutant strains shows robust reactivity to the P36 mAb. Immunoprecipitation experiments confirm that the P36 mAb interacts with the SRS9 protein. These data indicate that while the BSR4 protein is up-regulated in bradyzoites, the dominant antigen that the P36 mAb recognises is SRS9.

Keywords: *Toxoplasma gondii*; Surface antigens; P36 monoclonal antibody; SRS9; BSR4

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite capable of infecting virtually any nucleated cell (Miller et al., 1972). *T. gondii* has a sexual life cycle only in the feline intestinal epithelium and an asexual life cycle within all warm-blooded animals (Frenkel, 1985; Hutchison et al., 1970; Sheffield and Melton, 1970). The asexual cycle consists of two forms: the rapidly replicating tachyzoite and the slow-growing, encysted bradyzoite. Intermediate hosts such as humans can acquire *T. gondii* by ingestion of meat contaminated with bradyzoite cysts. Once inside the host, bradyzoites convert to tachyzoites, which are responsible for dissemination of the parasites until challenged by the host immune system. At this point, tachyzoites differentiate into encysted bradyzoites that remain dormant and can be hidden from the host immune system for years (Dubey et al., 1998). The biological triggers for differentiation from tachyzoites to bradyzoites remain unknown. In immunocompromised patients, such as those afflicted with acquired immunodeficiency syndrome (AIDS), bradyzoites can convert back to tachyzoites, leading to toxoplasmic encephalitis (Luft and Remington, 1992). Despite the importance of this developmental process, the molecular signals triggering interconversion between tachyzoites and bradyzoites are

* Corresponding author. Tel.: +1 608 262 3161; fax: +1 608 262 8418.
E-mail address: ljknoll@wisc.edu (L.J. Knoll).

0020-7519/$30.00
doi:10.1016/j.ijpara.2007.02.001

poorly understood. A molecular understanding of this reversible differentiation event will enhance our knowledge of *T. gondii* infections.

The SAG1-related sequence (SRS) proteins are a superfamily of glycosyl phosphatidyl inositol (GPI)-anchored surface antigens comprised of members of the SAG1 and SAG2 families (Nagel and Boothroyd, 1989; Tomavo et al., 1989). These families are structurally related and share conserved cysteine residues forming disulfide bonds (He et al., 2002), yet remain antigenically distinct (Jung et al., 2004). Members of the SAG1 family share 12 conserved cysteine residues and have an overall identity of approximately 30%, whereas members of the SAG2 family share only a subset of their cysteine residues and are less similar to each other with about 20% identity (Lekutis et al., 2001). As surface antigens, SAG1 and SAG2 are predicted to interact with host cells and may facilitate invasion, evasion of the immune system, or manipulation of host responses (Lekutis et al., 2001). Completion of the *T. gondii* genome project and identification of additional surface antigens has expanded the SAG1 and SAG2 families, all of which are now collectively named the SRS superfamily of proteins (Jung et al., 2004; Lekutis et al., 2001). Expression patterns vary amongst SRS proteins, although most are specific to either the tachyzoite or the bradyzoite stage (Jung et al., 2004). The biological importance of stage-specific expression of these surface antigens remains unknown.

To gain insight into the regulatory mechanisms controlling bradyzoite differentiation in *T. gondii*, antibodies against stage-specific markers for bradyzoites and tachyzoites were developed. A bradyzoite-specific mAb (T8 4A12), which recognises a 36 kDa surface antigen, was termed P36 (Tomavo et al., 1991). Since then the P36 mAb has been used frequently as a marker for bradyzoites in *T. gondii*, despite not knowing the genetic identity of this protein. Recently, an insertional mutant strain at the *BSR4* locus lost reactivity to the P36 mAb, suggesting that the BSR4 protein, a member of the SAG1 family, encodes the P36-reactive protein (Knoll and Boothroyd, 1998). Transient transfection of constitutively expressed *BSR4* restored P36 reactivity to this BSR4 insertional mutant as seen by immunofluorescence assay (IFA) (Knoll and Boothroyd, 1998). Surprisingly, the *BSR4* transcript was found to be equally abundant in tachyzoites and bradyzoites, suggesting post-transcriptional regulation of this gene (Knoll and Boothroyd, 1998). In the present study, complementation studies of BSR4 mutant strains with *BSR4* confirm a weak reactivity to the P36 mAb by Western immunoblotting. Investigation of other proteins detected by this antibody revealed SRS9 as the primary target of the P36 mAb. *SRS9* encodes a bradyzoite-specific protein with high similarity to members of the SRS family of surface antigens and is located immediately downstream of *BSR4*. Complementation and immunoprecipitation studies indicate that SRS9 is indeed the predominant protein recognised by the P36 mAb.

2. Materials and methods

2.1. Parasite growth and in vitro bradyzoite differentiation

Toxoplasma gondii type II strains Prugniaud (Pru) and Prugniaud deleted in hypoxanthine–xanthine–guanine phosphoribosyltransferase (PruΔHPT) were kindly provided by D. Soldati (University of Geneva). A derivative of PLK, another type II strain, in which the *BSR4* locus was interrupted with a promoterless *HPT* gene (Knoll and Boothroyd, 1998), will be referred to as *bsr4::HPT*. Tachyzoites were maintained by serial passage through human foreskin fibroblast (HFF) monolayers cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) (Invitrogen Life Technologies) at 37 °C with 5% CO_2. For bradyzoite induction, tachyzoites were allowed to infect HFFs for 3 h at 37 °C in 5% CO_2. Media were then replaced with RPMI 1640, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 8.0, 1% FBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), and cultures were incubated at 37 °C. After three days, bradyzoites were manually lysed from host cells by passage through a 27 gauge needle twice, once through a 30 gauge needle, and then collected by centrifugation at 423*g* for 10 min at room temperature.

2.2. Plasmids construction

The BSR4 deletion (ΔBSR4) construct was generated as follows. A 3.2 kb *Spe*I fragment comprised of sequences immediately upstream of the 5′ untranslated region (UTR) of *BSR4* was cloned into the *Spe*I site of pTgHXGPRTcI (Donald et al., 1996) in a tail-to-tail transcriptional orientation relative to *HPT*. A 1.9 kb fragment containing sequences downstream of the *BSR4* stop codon was amplified using the primers 5′-CTA AAGCTTTTCG TGTGTTCAACGTTTCC-3′ and 5′-CATAAGCTTGG TGGCTTACCACCAGTGAA-3′, generating *Hin*dIII sites (underlined) on both the 5′ and 3′ ends. The product was verified by sequencing, cloned into the *Hin*dIII site of the plasmid, in the reverse orientation relative to *HPT*.

For *BSR4* expression from the GRA1 promoter, the *BSR4* coding region was amplified using primers: 5′-GGGCATGCATGTGATGATGGGCAGCATGCA-3′ and 5′-GCCCTTAATTAATCACCAACCTACTCCCAA GGA-3′, adding *Nsi*I and *Pac*I sites (underlined), respectively. The product was verified by sequencing and cloned in replacement of the *NsiI-PacI GFP* fragment of pGRA1-GFP5S65T (Kim et al., 2001) to form pGRA1-BSR4. The pyrimethamine resistance marker DHFR was added to pGRA1-SRS9 (Kim and Boothroyd, 2005) and pGRA1-BSR4 by addition of a *Hin*dIII/*Spe*I fragment of DHFR-TSc3 (Donald and Roos, 1993) to the *Hin*dIII site of each plasmid by blunt ligation, creating pGRA1-SRS9-DHFR and pGRA1-BSR4-DHFR, respectively.