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A novel rhoptry protein in *Toxoplasma gondii* bradyzoites and merozoites

Jodi A. Schwarz[a,1,2], Ashley E. Fouts[a,2], Craig A. Cummings[a,b], David J.P. Ferguson[c], John C. Boothroyd[a,*]

[a] *Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305, USA*
[b] *VA Palo Alto Health Care System, Palo Alto, CA 94304, USA*
[c] *Nuffield Department of Pathology, Oxford University, John Radcliffe Hospital, Oxford, OX3 9DU, UK*

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Abstract

The secretory organelles of *Toxoplasma gondii* orchestrate invasion of the host cell and establish the parasitophorous vacuole. Although much has been learned about the roles played by these organelles in invasion by the tachyzoite stage, little is known about the contents or functions of these organelles during bradyzoite development or pathogenesis. We identified a novel protein that localizes to the rhoptries of the bradyzoite stage, but is absent from the tachyzoite stage. This protein, BRP1, first appears in the nascent rhoptries during the first division of bradyzoite stage development. We observed secretion of BRP1 and other rhoptry proteins into the parasitophorous vacuole during bradyzoite development in vitro, but there was no evidence that this occurs in vivo. Brp1 knockout parasites did not appear to have any developmental or growth defects in vitro, and were able to establish infections in mice both as tachyzoites (via intraperitoneal injection of in vitro-derived tachyzoites) or bradyzoites (via oral gavage using cysts harvested from mouse brain). Mice infected using brain cysts from the *brp1* knockout or the control strain developed similar numbers and sizes of brain cysts. Thus *BRP1* does not appear to play an essential role in development of the bradyzoite stage, development of brain cysts, or oral infection of new hosts, at least in the mouse model used here. Since we also observed that BRP1 is expressed in the merozoite stages in the gut of infected cats, the coccidian phase of the life cycle may be where BRP1 plays its most important role.

Keywords: *Toxoplasma gondii*; Bradyzoite; Roptry; Stage-specific; Merozoite

1. Introduction

The apicomplexan parasite, *Toxoplasma gondii*, is one of the most wide-ranging protozoan parasites of animals due to its ability to infect virtually any nucleated cell of warm-blooded animals, including humans. In humans, infection with *Toxoplasma* can cause severe disease, for example birth defects in individuals infected in utero or encephalitis in immunocompromised individuals. Symptoms of disease typically result from tissue damage caused by proliferation of tachyzoites. In the vast majority of hosts, however, infection is asymptomatic because the host's immune system clears tachyzoites from host tissues before extensive damage occurs.

The initial infection event occurs when oocysts or tissue cysts, ingested by a naïve host, rupture in the gut to release parasites that invade the intestinal epithelium [1]. Following the invasion event, parasites convert to tachyzoites that replicate rapidly and disseminate through the host. Host immune response causes tachyzoites to convert to cyst-forming bradyzoites that persist indefinitely within host tissues and are unaffected by the immune response. It is the development of a bradyzoite-containing tissue cyst that underlies the success of *Toxoplasma*, by allowing parasites to evade the host immune system and establish a life-long infection, and by facilitating transmission via carnivorism, which dramatically expands its host range [2–4].

Toxoplasma utilizes three different secretory organelles to effect control over the secretion of proteins and other molecules throughout the lytic cycle [5]. Micronemes are involved in invasion, while rhoptries discharge molecules required to establish the parasitophorous vacuole within the host cell. Dense granules secrete proteins into the parasitophorous vacuole throughout the

* Corresponding author. Tel.: +1 650 723 7984; fax: +1 650 723 6853.
E-mail address: john.boothroyd@stanford.edu (J.C. Boothroyd).
[1] Present address: Evolutionary Genomics Department, Joint Genome Institute, Walnut Creek, CA 94598, USA.
[2] JAS and AEF contributed equally to this work.

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lytic cycle, including stage-specific secretion of components of the bradyzoite cyst wall [6].

Although bradyzoites can be distinguished from tachyzoites morphologically, physiologically, and biochemically [7], it is not yet known to what degree there is stage-specific control over the contents and functions of the secretory organelles. For example, while the structure and function of the rhoptries have been extensively examined for the tachyzoite stage of parasite development, there is little information about the contents or functions of the rhoptries in other stages of the parasite's life cycle [8,9]. In particular it is not known whether this organelle contains components with functions that are specific to bradyzoite transmission, development, or pathogenesis.

To begin identifying parasite proteins that are likely to be secreted during the bradyzoite stage, we conducted a bioinformatic analysis of previously identified genes that are highly expressed during bradyzoite development [10] to predict which genes encode secretory proteins. This paper describes a protein identified by this method, a novel rhoptry protein of the bradyzoite stage, bradyzoite rhoptry protein 1 (BRP1).

2. Materials and methods

2.1. Parasite and host cell culture

Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle's medium (DMEM: Gibco) supplemented with 10% fetal bovine serum and 2 mM glutamine, penicillin, and streptomycin. *Toxoplasma gondii* tachyzoites (strain PruΔ*hpt* and derivatives) were maintained in confluent monolayers of HFFs. Conversion to the bradyzoite stage was effected by exchanging the media 4 h post-infection with RPMI1640 buffered with 50 mM HEPES to pH 8.15, and supplemented with 1% fetal bovine serum and antibiotics as described above.

2.2. Sequence analysis

To identify bradyzoite-specific secretory proteins, a bioinformatics analysis was performed using a dataset of bradyzoite-specific ESTs identified from a microarray study of bradyzoite development [10]. Of the 31 ESTs that had been demonstrated to be expressed at greater than two-fold higher in bradyzoites relative to tachyzoites (Fig. 3 from [10]), we selected the 18 for which a tblastx search of the nr databases with an *E* value threshold of 10^{-5} revealed no information regarding the potential function for the predicted peptide sequences. The EST sequence information (version 3 of the *Toxoplasma* EST database; http://paradb.cis.upenn.edu/toxo1/index.html) was used to map the 18 EST cluster sequences to the *Toxoplasma* genome (ToxoDB version 2.1, http://www.toxodb.org). ESTs were mapped to the genome and the overlapping predicted proteins were analyzed using SignalP [11,12] to detect potential signal peptides. Genomic data were provided by The Institute for Genomic Research (supported by the NIH grant #AI05093), and by the Sanger Center (Wellcome Trust). EST sequences were generated by Washington University (NIH grant #1R01AI045806-01A1). By these criteria, only one of the 18 ESTs mapped to an open reading frame that contained a predicted signal peptide. The full open reading frame was determined by examining genomic sequence, updated EST information in the apicomplexan EST database ApiDots ([13] http://www.cbil.upenn.edu/apidots), and by sequencing PCR products from cDNA libraries.

2.3. Production of recombinant protein and polyclonal antibodies

A DNA insert encoding the open reading frame of the predicted mature protein (amino acids 42–162) was prepared by PCR-amplification from a bradyzoite cDNA library and was cloned into the pCR2.1 vector (Invitrogen). The insert was excised from pCR2.1 and cloned into the pET28a expression vector (Novagen), such that a six-histidine tag would be synthesized at the C-terminal end. Transformation of *E. coli* BL-21pLys cells (Novagen) with the expression vector was performed according to the manufacturer's instructions. Protein expression was induced with IPTG and purification of the recombinant protein was performed with Ni-agarose beads (Qiagen).

The purified protein was dialyzed against PBS to prepare it for injection into mouse and rabbit. BALB/c mice were immunized intraperitoneally with approximately 50 μg of recombinant protein in 120 μl of phosphate-buffered saline (PBS) emulsified with an equal volume of RIBI adjuvant (RIBI Immunochem Research Inc.). Boosts were done at 2–3 weeks intervals. Blood was collected prior to initial immunization and after each boost from the tail vein, and the serum fraction was assayed by immunofluorescence assay (IFA). Rabbit polyclonal antibodies were prepared by Covance in a standard 118-day protocol using affinity-purified protein conjugated to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce) emulsified with RIBI adjuvant.

2.4. Indirect immunofluorescence assay

Tachyzoites or bradyzoites of all strains were grown in monolayers of HFFs on coverslips and processed for IFA [14]. Primary antibodies were used at the following dilutions: mouse polyclonal anti-recombinant protein 1:6000, rabbit polyclonal anti-recombinant protein 1:4000, monoclonal anti-ROP2,3,4 antibody (T3-4A71C8: gift from J.F. Dubremetz) 1:1500. Secondary antibodies (Molecular Probes Alexa fluor conjugated) were used at 1:2000 dilutions. The dolichos biflorus lectin conjugated to fluorescein was used at 1:700 dilution.

2.5. In vivo immunohistochemistry

Sections of cat small intestine containing the coccidian stages and mouse lung and brain containing the tachyzoite and bradyzoite stages of *T. gondii* were obtained as described previously [9]. For immunofluorescent staining, sections were pre-treated by pressure cooking and stained with mouse anti-BRP1 either alone or in combination with rabbit anti-ENO1, anti-ENO2, anti-NTPase, anti-SAG1 or anti-BAG1. After washing, sections were stained with goat anti-mouse immunoglobulin (Ig) conjugated to FITC and goat anti-rabbit Ig conjugated to Texas red. The