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Actin polymerization mediated by *Babesia gibsoni* aldolase is required for parasite invasion

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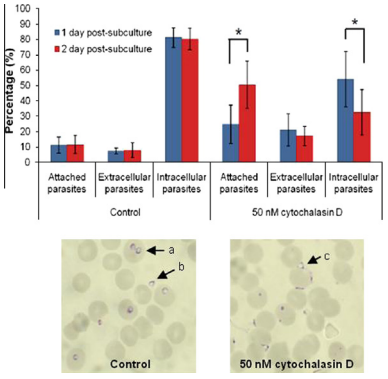
HIGHLIGHTS

- *B. gibsoni* aldolase forms a complex with *B. gibsoni* TRAP and *B. gibsoni* actin.
- The complex is likely to regulate an actin polymerization.
- Parasite growth is inhibited by treatment with cytochalasin D, an inhibitor of an actin polymerization.
- Actin dynamics seem to be essential for erythrocyte invasion by *B. gibsoni*.

GRAPHICAL ABSTRACT



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ABSTRACT

Host cell invasion by apicomplexan parasites driven by gliding motility and empowered by actin-based movement is essential for parasite survival and pathogenicity. The parasites share a conserved invasion process: actin-based motility led by the coordination of adhesin-cytoskeleton via aldolase. A number of studies of host cell invasion in the *Plasmodium* species and *Toxoplasma gondii* have been performed. However, the mechanisms of host cell invasion by *Babesia* species have not yet been studied. Here, we show that *Babesia gibsoni* aldolase (BgALD) forms a complex with *B. gibsoni* thrombospondin-related anonymous protein (BgTRAP) and *B. gibsoni* actin (BgACT), depending on tryptophan-734 (W-734) in BgTRAP. In addition, actin polymerization is mediated by BgALD. Moreover, cytochalasin D, which disrupts actin polymerization, suppressed *B. gibsoni* parasite growth and inhibited the host cell invasion by parasites, indicating that actin dynamics are essential for erythrocyte invasion by *B. gibsoni*. This study is the first molecular approach to determine the invasion mechanisms of *Babesia* species.

1. Introduction

The genus *Babesia* is an intraerythrocytic apicomplexan parasite that infects a wide variety of vertebrate hosts. *Babesia* infection causes serious economic losses in animals as well as life-threatening zoonotic infection (Homer et al., 2000; Hunfeld et al., 2008).

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Among the *Babesia* species, *Babesia gibsoni*, classified as a small *Babesia*, causes disease in Canidae characterized mainly by hemolytic anemia, fever, splenomegaly, and, occasionally, death (Boozer and Macintire, 2003). This organism was first recognized in India in 1910 and, since then, has been reported in Asia, Northern and Southern Africa, Brazil, Europe, and even Australia (Maronpot and Guindy, 1970; Matjila et al., 2007; Muhlnickel et al., 2002). Despite its widespread occurrence and clinical importance, proper vaccines and remedies have not been developed. *B. gibsoni* infection is transmitted naturally by the Ixodid tick. After a tick bite, the parasite is introduced into the bloodstream of a mammalian host and directly invades erythrocytes. Thereafter, the parasite replicates and egresses from the erythrocytes and then re-invades new erythrocytes. Of these steps, parasite invasion is the key process for the trigger of disease and pathogenicity (Homer et al., 2000). Therefore, a better understanding of the parasite invasion mechanisms would be very useful for the development of a vaccine and a drug for this disease.

Apicomplexan parasites invade the host cell by gliding motility, which is a unique substrate-dependent and actin-based locomotion (Baum et al., 2006a; Wetzel et al., 2003). Effective motility and invasion require molecules to be released from the secretory organelles, including micronemes, rhoptries, and dense granules. Among the molecules secreted from micronemes, thrombospondin-related anonymous protein (TRAP) and its orthologs of *Plasmodium* species and microneme protein 2 (MIC2) of *Toxoplasma gondii* contribute to parasite motility and invasion (Huynh and Carruthers, 2006; Morahan et al., 2009; Sultan et al., 1997). These adhesins are type-I transmembrane proteins that contain the thrombospondin type-I repeat (TSR) and/or von Willebrand factor (vWF) domain in their extracellular portion and a cytoplasmic tail (C-tail) domain. The binding of TSR and/or vWF domains with sulfated glycosaminoglycans on host cells and heparin-like molecules, respectively, is essential for cell attachment and invasion (Matuschewski et al., 2002; Wengelnik et al., 1999). Moreover, the C-tail of TRAP and MIC2 interacts with actin-binding aldolase in a tryptophan (W)-dependent manner, since indole ring of the penultimate W in C-tail of those adhesins interacts with the aldolase active site (Bosch et al., 2007; Buscaglia et al., 2003). In addition, the parasite with glycolytic enzyme aldolase mutation impairs anchoring between parasite adhesin and filamentous actins (F-actin) as well as host cell invasion (Jewett and Sibley, 2003). Previous studies have shown that treatment of the parasites with cytochalasins preclude host cell invasion and parasite motility in apicomplexan parasites, indicating that gliding motility relies on the formation of actin filaments (Dobrowolski and Sibley, 1996; Miller et al., 1979). In a eukaryotic cell, actin exists as a monomer bound to profilin. Thus, new actin filaments arise when signaling pathways activate nucleation-promoting factors and actin-regulatory proteins (Pollard and Borisy, 2003), indicating that the parasites require efficient control to assemble F-actin from the monomer (Dobrowolski et al., 1997; Field et al., 1993). Apicomplexan parasites lack genes for many known actin regulators, such as the Arp2/3 complex, which functions for *de novo* actin nucleation. Recently, formins were found to be a potent actin nucleator in *Plasmodium falciparum* and *T. gondii*, suggesting that actin dynamics may operate by alternative mechanisms in apicomplexan parasites (Baum et al., 2008; Daher et al., 2010). In a previous study, a recombinant TRAP of *B. gibsoni* (BgTRAP) was shown to bind erythrocytes, and specific antiserum to this protein suppressed parasite growth (Zhou et al., 2006a), suggesting that the BgTRAP may be the key element in the invasion step of *B. gibsoni*. In addition, BgACT was previously identified (Zhou et al., 2006b), and fructose-1,6-bisphosphate aldolase homologue was found in the EST database of *B. gibsoni*.

In this study, we show that *B. gibsoni* aldolase (BgALD) forms a complex with *B. gibsoni* TRAP (BgTRAP) and *B. gibsoni* actin (BgACT). In addition, the complex seems to regulate an actin polymerization. Moreover, parasite growth is inhibited by treatment with cytochalasin D, indicating that an actin dynamics are essential for erythrocyte invasion by *B. gibsoni*. Therefore, this study provides a new insight into the invasion mechanisms of *Babesia* species.

2. Materials and methods

2.1. Ethics statement

This study and all procedures were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval No. 23-72). The experimental animals were housed, fed, and given clean drinking water in accordance with the stipulated rules for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.2. Parasite cultures

The *B. gibsoni* Oita isolate was kindly provided by Dr. Sunaga, and an *in vitro* culture of *B. gibsoni*-infected RBC was maintained as described by Sunaga et al. (2002).

2.3. Cloning and expression of BgALD, BgTRAP, BgTRAP W/A, and BgACT

BgALD, BgTRAP, BgTRAPt, BgTRAP W/A, and BgACT were amplified from *B. gibsoni* cDNA using the primers in Table S1. The amplified PCR products were extracted using the Qiaquick gel extraction kit (QIAGEN, Germany) and then digested with *Bam*HI and *Xho*I. The digested DNAs were inserted between the *Bam*HI and *Xho*I sites of the expression vector pGEX-4T-1. Thereafter, the accurate insertions of the genes were confirmed by digestion with restriction enzymes and sequencing. The resulting plasmids having the gene inserts except BgTRAP gene were expressed as GST fusion proteins in the *Escherichia coli* BL21 (DE3) strain according to the manufacturer's instructions (GE Healthcare, UK). *E. coli* with plasmid including BgTRAP gene was incubated with 2% ethanol for 3 h at 37 °C, induced by 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for 10 h at 23 °C. Then, 400 ml of the resulting *E. coli* culture was centrifuged at 10,000 rpm for 10 min and then resuspended in 20 ml of a sodium–Tris–EDTA (STE) buffer (50 mM Tris–HCl (pH 9.0), 150 mM NaCl, 1 mM EDTA) containing 2 mg of lysozyme. The suspension of bacterial cultures was stirred for 1 h at 4 °C, sonicated on ice, and then centrifuged for 10 min at 10,000 rpm. The soluble proteins in the supernatant were bound to Glutathione-Sepharose beads (GE Healthcare, UK) equilibrated with phosphate buffer saline (PBS). Next, the beads were washed with 5 volumes of PBS with Tween-20 (PBST), and the GST-fused recombinant proteins were then eluted with 3 volumes of a 20 mM glutathione elution buffer (pH 8.0). The GST fusion proteins were digested with 1.0 units/μl of thrombin (GE Healthcare, UK) in PBS to obtain GST-cleaved proteins.

2.4. Production of anti-serum against BgALD, BgTRAP, and BgACT

Five 6-week-old female ICR mice (CLEA Japan, Inc., Japan) were intraperitoneally immunized with 200 μg of purified rBgALD, rBgTRAP, and rBgACT emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, USA). Two additional boosters with 100 μg of each antigen with incomplete Freund's adjuvant (Difco) were intraperitoneally administered at 2-week intervals. The mice were bled 14 days after the last booster, and serum samples were stored at −30 °C.