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NOTE

Interaction between *Theileria orientalis* 23-kDa piroplasm membrane protein and heparin

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Abstract

The 23-kDa piroplasm membrane protein of *Theileria orientalis* (p23) is an immunogenic protein expressed during the intraerythrocytic stage of the parasite; its function, however, remains unclear. To evaluate the host factor or factors that interact with p23, we examined the binding of p23 to components of the host cell surface. Recombinant p23 protein of the Ikeda genotype failed to bind to bovine red blood cells or to peripheral blood mononuclear cells, but did bind to Madin-Darby Bovine Kidney (MDBK) cells. A glycoarray assay showed that recombinant p23 proteins from the three genotypes bound to heparin, indicating that p23 is a heparin-binding *Theileria* surface molecule. Further analysis of heparin-binding molecules is useful for understanding attachment and invasion of *T. orientalis* merozoites.

Key Words: Glycoarray, Heparin, 23-kDa piroplasm membrane protein

Theileria orientalis is a tick-transmitted, intraerythrocytic protozoan belonging to the phylum Apicomplexa. It is a member of the nontransforming group of *Theileria* species (*Theileria sergenti/buffeli/orientalis*) that proliferates in the bovine host as an intraerythrocytic form[21)]. The *Theileria* species present in Japan, Korea, China, and Russia is locally known as *T. sergenti*[11,21)]. In Australia, this species is referred to as *T. buffeli*[5)]. *T. sergenti* is an invalid name taxonomically because it has been used previously to describe the parasite of sheep[22)]. *T. orientalis* is therefore commonly used to describe this benign *Theileria* group. Although *T. orientalis* is generally considered a relatively benign species, it can occasionally cause symptoms that include fever, anemia, and anorexia in infected cattle[19)]. The

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livestock industry in Japan suffers enormous economic losses due to bovine piroplasmosis caused by this parasite[16,18], because no effective medicine or vaccine is currently available for its control in Japan. In addition, *T. orientalis* disease outbreaks have caused substantial economic losses in other regions of the world[1,15].

Monoclonal antibodies against intraerythrocytic merozoites (piroplasms) of *T. orientalis* recognize major piroplasm surface protein (MPSP) and 23-kDa piroplasm membrane protein (p23)[14]. Both of these proteins are present on the surface of the merozoite membrane[20]. p23 has a signal peptide and a hydrophobic, valine-rich, putative transmembrane domain at its N- and C-terminus, respectively[17]. The N-terminal amino acid sequence of mature p23 has been determined[27]. Phylogenic analysis of the p23 gene indicates the presence of three distinct genotypes, namely Ikeda, Chitose, and Buffeli[16,17,26]. Ikeda type of *T. orientalis* is considered to be a relatively pathogenic genotype[8]. We previously demonstrated that recombinant MPSP binds to heparin and modified heparin derivatives (Takemae *et al.*, unpublished data); however, the molecular components that interact with p23 remain unknown. The interaction between p23 and the molecules of host cell surface is difficult to investigate experimentally, because no *T. orientalis* parasites have been adapted to *in vitro* culture.

Here, we examined the binding of recombinant p23 at the host cell surface. The coding sequences for p23 were amplified from previously isolated p23 gene fragments of the Ikeda, Chitose, and Buffeli genotypes[26] (The GenBank accession numbers are AB491353, AB491351, and AB491348, respectively) using the following primers: 5'-CAGCGCGGATCCGTACACACCTTG AAATCTGGC-3' (*Bam*HI site underlined) and 5'-ATAGTTTAGCGGCCGCCAAGAGAGGCAAC AACAACG-3' (*Not*I site underlined). The amplified p23 gene fragments of three genotypes were cloned into the *Bam*HI/*Not*I sites of the pGEX-4T-1 plasmid. To assess binding to the host cell surface, we prepared recombinant p23 protein of the Ikeda genotype in insect cells. The coding sequence for p23 was amplified from a pGEX-4T-1 plasmid containing the p23 gene fragment of the Ikeda genotype by using the KOD FX Neo PCR enzyme (Toyobo, Osaka, Japan) with the following primers: 5'-GCGGATCCGATATCGAC AAGGATGG-3' (*Bam*HI site underlined) and 5'-T CCCCCGGGGTCAGACTTCTCTTCCTCAGTA-3' (*Sma*I site underlined). The amplified fragment was then cloned into the *Bam*HI/*Sma*I sites of the baculovirus expression vector pBSV-Fc-8His[12] to express a fusion protein with the Fc region of murine IgG2a at the N terminus and an octahistidine tag at the C terminus. The baculovirus expression plasmid was co-transfected with BaculoGold DNA (BD Biosciences, San Jose, CA) into Sf9 insect cells. Amplification of recombinant baculovirus and protein purification were performed as described previously[12]. The purified Fc-p23-8His recombinant protein produced multiple bands on SDS-PAGE (Fig. 1A, *lane 4*). We used this recombinant protein for binding experiments because immunoblotting with an anti-mouse Fc antibody detected an approximately 50-kDa band similar to the theoretical molecular weight of Fc-p23-8His (47.9 kDa) (Fig. 1A, *lane 6*). To evaluate p23 binding to the cell surface, we examined the binding of the Fc-p23-8His protein to bovine red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs), and Madin-Darby Bovine Kidney (MDBK) cells. Bovine PBMCs were isolated from bovine whole blood by centrifugation through Histopaque 1077 (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. The recovered PBMCs were cryogenically preserved as described previously[25]. Monolayers of MDBK cells were washed with PBS and then dispersed with 0.02% EDTA-4Na in PBS at 37°C. RBCs, PBMCs, and MDBK cells (10^5 cells each) were washed once in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FCS and 0.1% NaN_3) and incubated with 2 pmol of Fc-8His or Fc-p23-8His

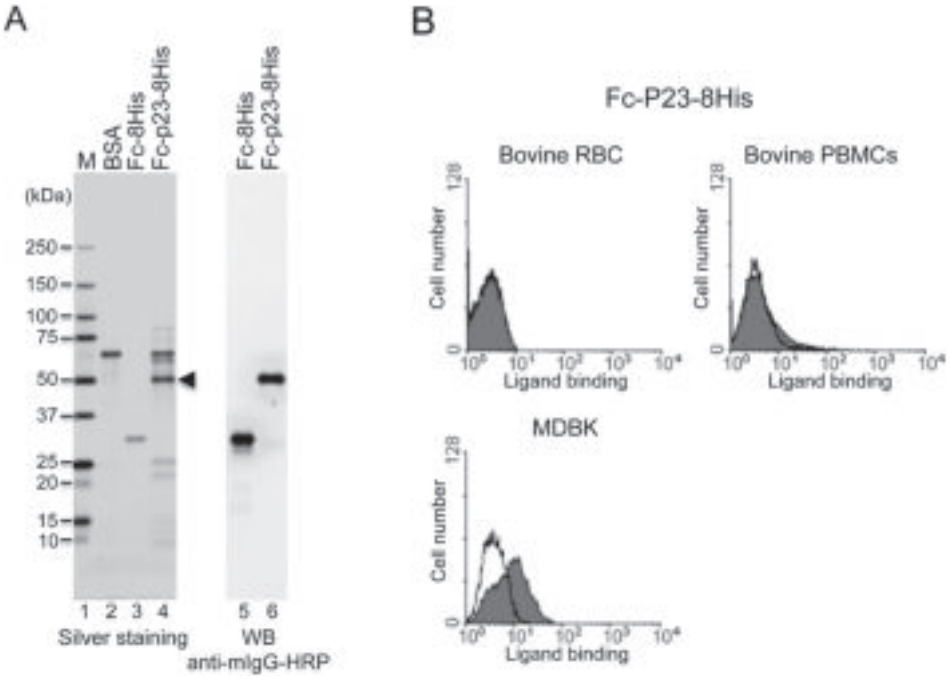

Fig. 1. Binding of recombinant p23 to the cell surface. (A) Recombinant proteins expressed using the baculovirus expression system and purified on Ni-NTA agarose. BSA, Fc-8His, and Fc-p23-8His recombinant proteins (20 ng) were separated by 5%–20% gradient SDS-PAGE and subjected to silver staining (lanes 2–4). The molecular masses (kDa) are indicated on the left. Immunoblotting of purified recombinant proteins with an HRP-conjugated anti-mouse Fc antibody (lanes 5, 6). Lane M is a molecular weight marker. The arrowhead indicates a 50-kDa band that is equivalent to Fc-p23-8His. (B) Bovine RBCs, bovine PBMCs, and MDBK cells were incubated with 2 pmol each of Fc-p23-8His from the Ikeda genotype (shaded area) or Fc-8His (solid line), and then incubated with an FITC-conjugated anti-mouse Fc antibody. Binding to the cell surface was detected by use of flow cytometry.

in FACS buffer for 1 h at 4°C. The cells were then washed twice and incubated for 20 min on ice with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA). After the cells were washed with FACS buffer, antibody binding was quantitated on a FACSCalibur (BD Biosciences). The Fc-p23-8His recombinant protein did not bind to either the RBCs or the PBMCs (Fig. 1B). The p23 protein, however, bound to MDBK cells (Fig. 1B). These results suggest that p23 has binding affinity for some components on the cell surface.

To further investigate whether recombinant p23 proteins bind to bovine RBCs or not, an RBC binding assay was performed as previously described with modification[9]. To compare binding property among p23 proteins from different genotypes, we prepared GST-fusion recombinant p23 proteins (GST-p23) from the Ikeda, Chitose, and Buffeli genotypes. These proteins were expressed in *E. coli* strain BL21 (DE3) pLysS and purified by using the MagneGST™ protein purification system (Promega, Madison, WI, USA). The purified GST-p23 recombinant protein produced a major band on SDS-PAGE with a molecular mass of approximately 45 kDa, similar to its predicted size of 49.7 kDa (Fig. 2A, *lanes 3–5*). Five hundred ng of GST or GST-p23 recombinant protein was mixed with 50 µl of packed bovine RBCs and incubated in binding buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MnCl_2$; 1 mM $MgCl_2$) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) for 2 h at 4°C. After centrifugation, the supernatant containing unbound protein was pooled. The pelleted bovine RBCs was resuspended in 500 µl of culture medium and layered onto 500 µl of

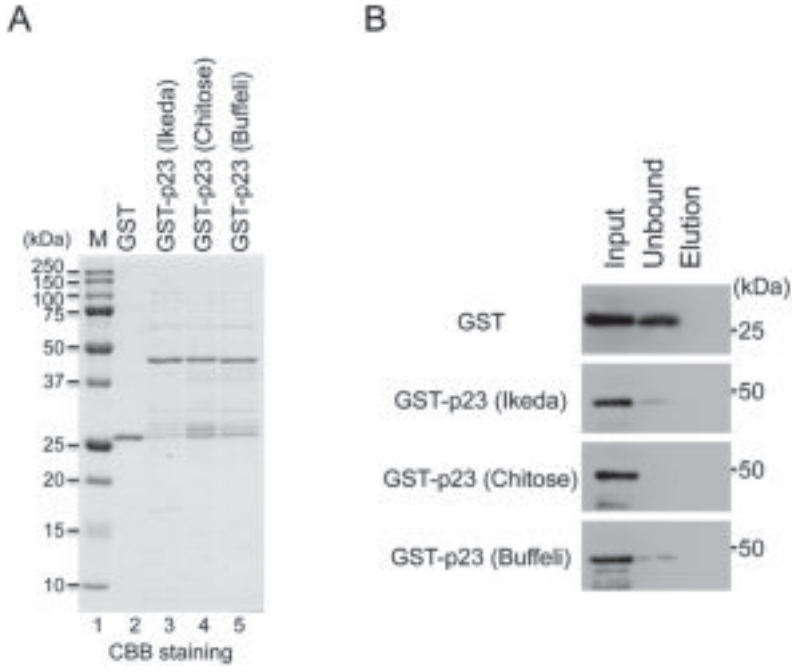

Fig. 2. RBC binding analysis of p23 recombinant proteins. (A) Bacterially expressed GST and GST-p23 recombinant proteins from three different genotypes (Ikeda, Chitose, and Buffeli) were purified by using Glutathione-Sepharose 4B beads. Each recombinant protein (200 ng) was separated by 12.5% SDS-PAGE and subjected to CBB staining (lanes 2–5). (B) 0.5 μg each of GST, GST-p23 (Ikeda, Chitose, and Buffeli) recombinant protein was incubated with packed bovine RBCs. After the unbound protein was separated by centrifugation of the bovine RBCs layered onto silicon oil, the bound protein was eluted with 1.5 M NaCl and purified by using glutathione beads. The input (20% per total volume), unbound (20% per total volume), and eluted proteins were analyzed by Western blotting with an anti-GST antibody.

silicon oil (SIGMA-Aldrich), and then centrifuged at 15,000 x*g* for 1 min. The bovine RBCs were recovered from the bottom of tube, washed with PBS and bound GST-p23 recombinant proteins were eluted in 50 μl of 1.5 M NaCl. The eluate was purified by using the MagneGST™ Glutathione Particles. GST-p23 recombinant proteins were not detected in elution fraction (Fig. 2B). These proteins were not also recovered from the unbound fraction, whereas almost all of control GST was recovered from the unbound fraction (Fig. 2B). This result showed that recombinant p23 proteins do not bind to bovine RBCs under our experimental conditions. The possibility remains that GST-p23 recombinant proteins is degraded by contacting with bovine RBCs, probably in a protease independent manner.

The interaction of p23 with various oligosaccharides was investigated by using an *in vitro* glycoarray system. The glycoarray plate BS-X1707 (Sumitomo Bakelite, Tokyo, Japan) was incubated with 1 μg of GST or GST-p23 recombinant protein in reaction buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MnCl_2$; 1 mM $MgCl_2$; 0.05% Tween 20; 10 mg/ml BSA) for 16 h at 4°C, washed with wash buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MnCl_2$; 1 mM $MgCl_2$), and then incubated with 10 μg/ml of anti-GST antibody (Medical & Biological Laboratories, Nagoya, Japan) in reaction buffer for 16 h at 4°C. The secondary antibody reaction was performed using 10 μg/ml of Alexa Fluor 546 goat anti-mouse IgG in reaction buffer for 1 h at 25°C. The plate was then scanned with a ScanArray Lite (PerlinElmer, Waltham, MA). The fluorescent signal of GST-p23 was detected at the spot corresponding to heparin and at a faint spot corresponding to a 2-*O*-desulfated heparin

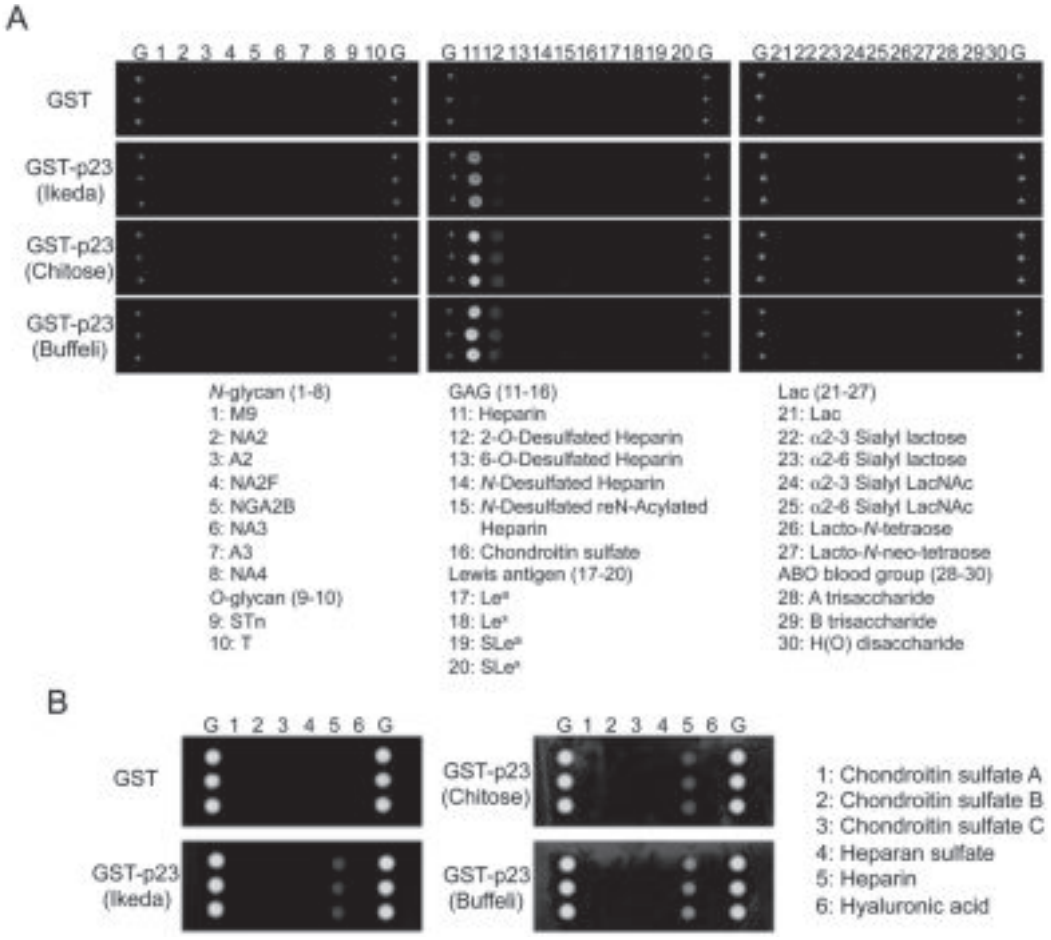

Fig. 3. Glycoarray analysis. (A, B) GST, GST-p23 (Ikeda), GST-p23 (Chitose), or GST-p23 (Buffeli) recombinant protein (1 μg each) was incubated with the glycoarray plate BS-X1707 (A) or the glycosaminoglycan array plate (B). The plate was then incubated with an anti-GST antibody. Alexa Fluor 546 goat anti-mouse IgG served as the secondary antibody. The fluorescence intensity of the plate-bound protein was measured with a fluorescence scanner. The oligosaccharides spotted on the glycoarray plate are listed. G shows the grid marker.

derivative (Fig. 3A); the control GST did not react with these oligosaccharides (Fig. 3A, *11, 12*). GST-p23 did not bind to any other desulfated heparin derivatives tested (Fig. 3A, *13–15*) or to chondroitin sulfate (chondroitin sulfate A) (Fig. 3A, *16*), *N*-glycans, *O*-glycans, Lewis antigens, lactose derivatives, or ABO blood group antigens. The recombinant proteins from the Ikeda, Chitose, and Buffeli genotypes showed almost same binding patterns (Fig. 3A). To validate the binding specificity between p23 and heparin, we investigated the interaction of p23 with various glycosaminoglycans by using another glycoarray plate (Sumitomo Bakelite, Tokyo, Japan) (Fig. 3B). The GST-p23 recombinant proteins from the three different genotypes we tested bound to heparin, but not to chondroitin sulfates, heparan sulfate, or hyaluronic acid. These results demonstrate that p23 binds to heparin and that this binding is not affected by the different genotypes of p23.

In this study, we searched for host cell components that bind to *T. orientalis* p23 and identified heparin as a binding component of p23. There have been few previous reports concerning the host factors that interact with *T. orientalis* proteins, with the exception of a study showing that ToMRP, a *T. orientalis* piroplasm protein related to p104 microneme-rhoptry protein in *Theileria parva*, interacts with band 3

protein of bovine erythrocytes[10]. We previously showed that recombinant MPSP protein binds to heparin and to modified heparin derivatives (Takemae *et al.*, unpublished data). The MPSP proteins bear at least one potential GAG (glycosaminoglycan)-binding motif, characterized by XBBXBX and XBBBXXBX (where B is lysine or arginine and X is any other amino acid)[6], whereas p23 proteins have known no GAG-binding motif. These data are consistent with the result of our glycoarray assay in which recombinant p23 proteins from the Ikeda, Chitose and Buffeli genotypes all bound only to heparin (Fig. 3).

Heparin is a polysaccharide consisting of repeating disaccharide units of an uronic acid molecule and a glucosamine molecule. It is a highly sulfated form of heparan sulfate. Heparin and heparan sulfate can block the invasion of erythrocytes by *Plasmodium falciparum* merozoites and cytoadherence of parasitized erythrocytes to endothelial cells[24]. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and merozoite surface protein 1 (MSP1) are thought to be the targets for heparin and heparan sulfate in rosette disruption, desequestration, and invasion inhibition[4,23]. In *Babesia bovis*, fluorescein isothiocyanate (FITC)-labelled heparin is preferentially found on the surface of extracellular merozoites[3]. Specific sulfated glycoconjugates, such as dextran sulfate, heparin, heparan sulfate, fucoidan, and chondroitin sulfate B strongly inhibit the asexual growth of *B. bovis* merozoites[2]. Heparin also inhibits the invasion of host RBCs by *T. orientalis* merozoites in a dose-dependent manner[7]. Hemagglutination of bovine RBCs by *T. orientalis* merozoites is inhibited by heparin, but this activity is observed even after treatment of bovine RBCs with heparinase, suggesting that heparin-like molecules on bovine RBCs might not have a role in invasion by *T. orientalis* merozoites[7]. Our recent study has shown that the binding of PfMSP1 to heparin is specific, but relatively weak[13]. Although recombinant p23 proteins did not bind to bovine RBCs under our experimental conditions, these proteins may be degraded by erythrocyte factor. The possibility remains that binding affinity may be different between *in vivo* and *in vitro* because the level of sulfation of heparan sulfate on the cell surface is different. The binding of p23 to MDBK cells may contribute to some components except for heparin on the cell surface. Our study showed that p23 is a heparin-binding *Theileria* surface molecule. Further studies of other heparin-binding molecules will provide a better understanding of *T. orientalis* attachment and invasion.

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