



Toxoplasma gondii RON4 binds to heparan sulfate on the host cell surface

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ABSTRACT

Toxoplasma gondii rhoptry neck protein 4 (TgRON4) is a component of the moving junction, a key structure for host cell invasion. We previously showed that host cellular β -tubulin is a binding partner of TgRON4 in the invasion process. Here, to identify other binding partners of TgRON4 in the host cell, we examined the binding of TgRON4 to components of the host cell surface. TgRON4 binds to various mammalian cells, but this binding disappeared in glycosaminoglycan- and heparan sulfate-deficient CHO cells and after heparitinase treatment of mammalian cells. The C-terminal half of TgRON4 showed relatively strong binding to cells and heparin agarose. A glycoarray assay indicated that TgRON4 binds to heparin and modified heparin derivatives. Immunoprecipitation of *T. gondii*-infected CHO cell lysates showed that TgRON4 interacts with glypican 1 during *Toxoplasma* invasion. This interaction suggests a role for heparan sulfate in parasite invasion.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite of the phylum Apicomplexa, which includes the human and animal parasites *Plasmodium*, *Eimeria*, *Neospora*, *Theileria*, *Babesia*, and *Cryptosporidium*. It is estimated that approximately one-third of humans worldwide are chronically infected with *T. gondii* [1,2], which can cause toxoplasmosis in immunocompromised individuals and neonates with congenital infections [3].

Toxoplasma gondii RON4 (TgRON4) is one of the components of the tight junction structure known as the moving junction; moving junctions are central structures formed by close opposition of the invading parasite and the host cell membranes during invasion. The parasite propels itself by means of an internal actomyosin motor, thereby leading to the formation of a parasitophorous vacuole [4]. In *T. gondii*, the moving junction consists of TgRON2, TgRON4, TgRON5, and TgRON8 secreted from rhoptry and AMA1 secreted from micromere [5–9]. TgRON2 is inserted as an integral transmembrane protein into the host plasma membrane, whereas TgRON4, TgRON5, and TgRON8 are exposed to the cytosolic face of the host cell membrane during invasion [6]. Components of this complex are conserved among Apicomplexan species [10–12], except for TgRON8, which seems to be

coccidian-specific [13]. From the study of TgRON8-knockout parasites, it has been proposed that TgRON8 forms a firm intracellular grip that commits the parasite to invasion by anchoring it to the host cytoskeleton [8]. A TgRON5 conditional knockout strain demonstrated that TgRON5 is required for TgRON2 stability and the proper targeting of TgRON4 [14]. Lamarque et al. [15] showed that TgRON4 and TgRON5 are not detected at the moving junction during invasion on a TgRON2 conditional knockout parasite. In addition to the importance of TgRON2 and TgRON5, TgRON4 is thought to be crucial for parasite growth as attempts to knock it out were unsuccessful in *T. gondii* tachyzoites [5].

We previously demonstrated that TgRON4 binds to host cellular β -tubulin and that the TgRON4-binding region of β -tubulin colocalizes with TgRON4 in *T. gondii*-infected mammalian cells [16]. These findings suggest that soluble TgRON4 and TgRON8 exported to the cytosolic side of the host cell could associate with the cortical host cytoskeleton. After invasion is completed, the moving junction can still be detected at the posterior pole of the parasite for a few hours [17]. In intracellular parasites, TgRON4 is detected in the rhoptry neck, as well as in the parasitophorous vacuole [5,17,18], but TgRON5 and TgRON8 localize to only the rhoptry neck [6]. TgRON4, but not TgAMA1, is associated with the moving junction during ionophore-induced egress

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[5]. Moreover, TgRON4 shows a typical ring-like signal in conditional TgAMA1 null mutant tachyzoites, indicating that TgAMA1 is not essential for the formation of a functional moving junction [19]. Together, these results imply that TgRON4 may function independently of AMA1 and/or other soluble RON proteins.

In this study, we investigated the binding of TgRON4 to components of the host cell surface. A binding assay using various CHO mutant cells indicated that heparan sulfate is required for binding to TgRON4. We also show that TgRON4 recombinant protein binds directly to heparin and sulfation on glucosamine of heparan sulfate is important for its binding to TgRON4. By using immunoprecipitation assay, we also showed that TgRON4 interacts with glypican 1.

2. Materials and methods

2.1. Cell culture

Wild-type CHO, pgsA-745 (American Type Culture Collection (ATCC), CRL-2242) [20], pgsD-677 (CRL-2244) [20], pgsE-606 (CRL-2246), Lec2 (CRL-1736), and ldlD (ATCC, SD-1401) cells were maintained in Ham F-12 medium (Thermo Fisher Scientific, Waltham, MA) with 10% FCS and penicillin and streptomycin. 293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% FCS, glutamine, penicillin and streptomycin. Vero cells were cultured in DMEM with 5% FCS. Jurkat-EcoVRc [21,22], and K562-EcoVRc cells (kindly provided by Dr. Masayuki Shimojima) were maintained in RPMI 1640 medium (SIGMA) supplemented with 10% FCS. These latter cells are Moloney murine leukemia virus (MMLV)-sensitive human cells that express the mouse ecotropic retrovirus receptor (EcoVRc) and were prepared by using a self-inactivating human immunodeficiency virus vector [23]. *Spodoptera frugiperda* Sf9 and *Trichoplusia ni* Tn5 insect cells were cultured in SF900II SFM (Thermo Fisher Scientific) supplemented with 10% FCS and EX-CELL 405 (SAFC Biosciences Inc., Lenexa, KS), respectively.

2.2. Preparation of TgRON4 recombinant proteins

Fc-8His, Fc-TgRON4 (Full)-8His, and 3xFLAG-TgRON4-8His recombinant proteins were prepared as described previously [16]. The coding sequences for the N-terminal (amino acids 29–500) and C-terminal (amino acids 481–984) halves of TgRON4 were amplified from the Fc-TgRON4-8His plasmid by using KOD FX Neo PCR enzyme (Toyobo, Osaka, Japan) with a forward primer containing a *SpeI* site and a reverse primer containing a *SmaI* site. These amplified fragments were cloned into the *SpeI/SmaI* sites of the baculovirus expression vector pBSV-Fc-8His [22]. The resultant plasmids were designated pBSV-Fc-TgRON4A-8His and pBSV-Fc-TgRON4B-8His, respectively. To generate the pBSV-3xFLAG-RON4A- and -RON4B-8His vectors, each TgRON4 cDNA was cloned into the *SpeI/SmaI* sites of pBSV-3xFLAG-8His [16].

The pBSV-GST-3xFLAG-8His vector was designed to express GST-3xFLAG-8His recombinant protein in insect cells. To generate the pBSV-MCS-3xFLAG-8His vector, a fragment containing the 3xFLAG sequence was amplified by PCR from pBSV-3xFLAG-8His with the following primers: 5'-CGAATTCTCCCGGGGACTACAAAGACCATGACG-3' (*EcoRI* site underlined) and 5'-CTTGTCATCGTCATCCTTGTA-3'. The PCR product was digested with *EcoRI* and then ligated to the *EcoRI/SmaI* sites of pBSV-8His [24]. A fragment coding GST was amplified by PCR using the pGEX-6P-2 vector (GE healthcare) as the template with the following primers: 5'-AACTGCAGTCCCCTATACTAGGTTATTGGA-3' (*PstI* site underlined) and 5'-CGGGATCCGAGGGGCCCTGGAA-3' (*BamHI* site underlined). The amplified fragment was cloned into the *PstI/BamHI* sites of pBSV-MCS-3xFLAG-8His.

These plasmids were 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 [22]. The culture supernatant was used for Ni-NTA agarose chromatography. A small aliquot of elution fraction with imidazole (50 mM to 300 mM) was examined using anti-His antibody. Fractions positive for the antibody were pooled and concentrated with ultrafiltration.

2.3. Flow cytometry

Monolayers of wild-type CHO or various CHO mutant cells were dispersed with 0.02% EDTA-4Na in PBS. Cells (10^5 cells) were washed once in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FCS and 0.1% NaN_3) and incubated with 0.5 pmol of Fc-8His or Fc-TgRON4 Full-8His 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). After the cells were washed with FACS buffer, antibody binding was quantitated on a FACSCalibur (BD Biosciences).

In the heparitinase treatment experiment, 293 T or Vero cells (3×10^5) were incubated in PBS containing 1 mM CaCl_2 and 0.05 mM MgCl_2 and 0.05% BSA in the presence or absence of 0.02 U/ml heparitinase from *Flavobacterium heparinum* (Seikagaku Corp., Tokyo, Japan) for 1 h at 37 °C. After being washed with FACS buffer twice, the heparitinase-treated or -untreated cells (10^5 cells) were incubated with 0.5 pmol of Fc-8His, Fc-TgRON4A-8His, or Fc-TgRON4B-8His in FACS buffer for 1 h at 4 °C. Binding of the recombinant proteins was detected as described above.

2.4. Expression of heparan sulfate proteoglycan (HSPG) core proteins by retroviral gene transfer

Jurkat-EcoVRc and K562-EcoVRc cells expressing glypican 1, glypican 4, syndecan 1, or syndecan 4 were prepared by transducing these human cells with a retroviral vector encoding each heparan sulfate proteoglycan core protein, as previously described [22]. In brief, a retroviral vector containing pMX-glypican or pMX-syndecan was produced in a Plat-E packaging cell line. Two days after transfection of the retroviral vectors, the supernatants containing ecotropic virus were collected and filtered through a 0.45 μm filter. Jurkat-EcoVRc and K562-EcoVRc cells were incubated with the virus supernatant for 8 h and then used for flow cytometry. Binding of the TgRON4 recombinant protein was detected as described above.

2.5. Binding assay to heparin agarose

GST-3FLAG-8His or 3FLAG-TgRON4-8His recombinant protein (200 ng) was incubated with a 20- μl suspension of heparin agarose (SIGMA) in NETT buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.02% NaN_3) containing 0.5% BSA and Complete EDTA-free protease inhibitor cocktail (Roche) for 1 h at 4 °C with rotation. The beads were washed three times with NETT buffer and then boiled for 5 min in equal volumes of 2 x SDS sample buffer (0.1 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT). The eluates were separated by 5%–20% gradient SDS-PAGE (Atto, Tokyo, Japan) for Western blot analysis using an anti-His (Applied Biological Materials, Richmond, BC) antibody. Band intensities of the unbound and elution fractions were quantified using ImageJ software.

2.6. Glycoarray assay

The glycoarray plate BS-X1707 (Sumitomo Bakelite, Tokyo, Japan) was incubated with 7.5 μg of Fc-8His or Fc-TgRON4 Full-8His 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, and then incubated with 10 $\mu\text{g}/\text{ml}$ of the secondary antibody Alexa Fluor 546 goat anti-mouse IgG in reaction buffer for 1 h at 25 °C. The plate was scanned with a

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