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TgGRA23, a novel *Toxoplasma gondii* dense granule protein associated with the parasitophorous vacuole membrane and intravacuolar network

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ABSTRACT

Toxoplasma gondii is an intracellular protozoan parasite, which relies on a specialized compartment, the parasitophorous vacuole (PV), to survive within host cells. Dense granules within the parasite release a large variety of proteins to maintain the integrity of the vacuole structure. Here, we identified a novel dense granule protein in *T. gondii*, TgGRA23, which is a homolog of the *Sarcocystis muris* dense granule protein, SmDG32. Recombinant TgGRA23 (rTgGRA23) expressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein was used to raise antisera in mice and rabbits. Immunoblotting showed that antisera from the immunized mice and rabbits reacted with parasite lysates to yield a 21-kDa native protein. In addition, immuno-electron microscopic examination showed that TgGRA23 resides in the dense granules, PV membrane and intravacuolar network of the parasite. To confirm the precise subcellular localization of TgGRA23 in *T. gondii*, an immunofluorescent antibody test was performed using dense granule markers. Notably, TgGRA23 co-localized with other dense granule proteins including TgGRA4 and TgGRA7, in the extracellular-stage parasites. Biochemical experiments indicated that TgGRA23 is insoluble and may form an electrostatic complex that is resistant to non-ionic detergents. Furthermore, specific antibodies to TgGRA23 were detected during the chronic stage of *Toxoplasma* infection in mice. Our results suggest that TgGRA23 is an as yet unknown member of the *T. gondii* dense granule proteins, and that it may be involved in remodeling or maintenance of the PV.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa, which is capable of infecting a broad range of warm-blooded animals, including humans. The infection is usually asymptomatic in immunocompetent hosts, but can be potentially fatal in immunocompromised individuals [1]. Infection during pregnancy may result in abortion, weak offspring, stillbirth and birth defects. Moreover, ocular diseases have been reported in patients who were congenitally or postnatally infected [2]. The pathogenesis of *T. gondii* is due to the rapid replication of tachyzoites in the host cells, which results in their lysis. Successful intracellular development of tachyzoites requires a safe sub-cellular compartment within the host cells, a structure which is called the parasitophorous vacuole (PV) [3]. As with other Apicomplexan parasites, *T. gondii* has specialized secretory organelles (such as micronemes, rhoptries and dense granules), which are thought to perform the many different functions required for successful host cell invasion, formation of the PV and establishing infection. First, microneme proteins are released upon contact with a host cell; these serve in recognition and attachment of the parasite [4]. Rhoptry proteins are released next, and their function is required for the formation of the nascent PV [5]. Thereafter, dense granule proteins are secreted from the parasites into the PV where they serve in the remodeling and maintenance of this compartment [6]. Members of the *T. gondii* dense granule protein family (GRA1-GRA10, GRA12, GRA14, GRA15, GRA19-21, CyP18, TgPIs, TgNTPs, Tg14-3-3) [7–9] are known to participate in modification of the PV and PV membrane (PVM), which is an essential process for maintenance of intracellular parasitism of host cells. Antibodies against several dense granule proteins [10–16] have been used as serological markers of acute infection in both humans and animals; hence dense granules are potentially useful diagnostic markers. Therefore, identification and characterization of dense granule proteins would be an important step towards better understanding of the biology of *T. gondii*. In addition, the knowledge of the proteins may assist the development of effective chemotherapeutic agents against this pathogen. In this study, a novel dense granule *T. gondii* protein that shares some

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homology with the 32-kDa dense granule protein of the coccidian parasite *S. muris* [17] was identified. Characterization of this protein, hereafter designated TgGRA23, revealed that it is localized in the dense granules, at the PVM and at the intravacuolar network of the parasite.

2. Materials and methods

2.1. Cells and parasites

Vero cells were grown in minimum essential medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified MEM (Sigma) supplemented with 10% heat-inactivated FCS, 100 U/ml of penicillin and 100 μg/ml streptomycin. The *T. gondii* RH strain (American Type Culture Collection, Catalog No. 50174) and PLK strain (American Type Culture Collection, Catalog No. 50841) were maintained in a monolayer of either Vero cells or HFF cells.

2.2. Cloning of the gene encoding TgGRA23

T. gondii PLK strain tachyzoites were purified from host cells using 27-gauge needles and 5-μm pore size filters, Millex-SV (Millipore, Billerica, MA, USA). Total RNA from the parasites was prepared using TRI reagent (Sigma). Parasite cDNA was synthesized from PLK total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The complete sequence of the gene encoding TgGRA23 (TGME49_297880 in ToxoDB 8.0 [http://toxodb.org/toxo/]) was amplified using KOD plus-Neo PCR enzyme (TOYOBO, Osaka, Japan) with the following set of primers: forward (5'-CA*A TG*G CAG CGC GTG CGG GAA G-3') and reverse (5′-CT*C TA*G TTC TTT CGC GCA AGG GG-3′) (start and stop codons are underlined). The PCR product was cloned into a T-Vector pMD20 (Takara Bio, Inc., Shiga, Japan), and the gene sequence was analyzed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

2.3. Expression and purification of recombinant TgGRA23 (rTgGRA23)

The cDNA encoding TgGRA23 (lacking its signal peptide sequence) was amplified from the pMD20-cloned TgGRA23 sequence using the following set of primers: w/o-SP forward (5′-CTC GAA TTC GAC GAC GCC TTC ATA GAC AAT-3′) and w/o-SP reverse (5′-CTC TCG AGC TAG TTC TTT CGC GCA AGG GG-3′) (the underlined sequences are *Eco*RI and *Xho*I sites, respectively). The PCR product was digested with *Eco*RI and *Xho*I and then ligated to a similarly cut pGEX-4T1 vector containing an open reading frame (ORF) encoding glutathione *S*-transferase (GST) fused to the N-terminus of the protein (GE Healthcare, Little Chalfont, UK) using a DNA Ligation Kit Mighty Mix (Takara Bio, Inc.). After verification of the proper in-frame position of the sequence, the recombinant plasmid was used to transform *E. coli* (BL21) cells. The recombinant TgGRA23 (rTgGRA23) was expressed as a GST-fusion protein (rTgGRA23-GST) in *E. coli* and purified using Glutathione-Sepharose 4B beads (GE Healthcare) as previously described [18]. Digestion of the GST moiety was performed using thrombin (GE Healthcare), which was removed by treatment with benzamidine sepharose (GE Healthcare). The rTgGRA23 protein was dialyzed in PBS, and its concentration was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Expression of the recombinant protein was confirmed by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel.

2.4. Construction and expression of recombinant TgGRA7 (rTgGRA7)

cDNA was synthesized from total RNA from the *T. gondii* RH strain following isolation with TRI reagent using the SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA). To clone the 232 amino acid recombinant TgGRA7 without the signal peptide (amino acid 1 to 22), a set of primers that included a BamHI site in the forward primer (5′-GCG GAT CCG CCA CCG CGT CAG ATG-3′) and an EcoRI site in the reverse primer (5′-GTG AAT TCC TAC TGG CGG GCA TCC TC-3′) (the underlined sequences are the *Bam*HI site and *Eco*RI site, respectively) was designed. The PCR product was digested with *Bam*HI and *Eco*RI and then ligated to a pGEX-4T3 vector containing an ORF encoding GST fused to the N-terminus of the protein (GE Healthcare). rTgGRA7 was expressed as a GST-fusion protein in *E. coli*, purified with Glutathione-Sepharose 4B beads and dialyzed in PBS. The protein concentration was measured using a BCA protein assay kit.

2.5. Antisera

Mouse sera against rTgGRA4 [19] and rTgAMA1 [20,21] and rabbit sera against rTgGRA1 [22,23] were raised and used as described previously. The anti-TgSAG1 mouse monoclonal antibody was purchased from Advanced ImmunoChemical Inc. (Long Beach, CA, USA). Serum against rTgGRA23 was induced in mice as follows: one hundred micrograms of rTgGRA23 was injected intraperitoneally into ICR mice (6-week-old, female, CLEA Japan, Tokyo, Japan) with Freund's complete adjuvant (Sigma). On days 14 and 28 post-immunization, the same antigen was intraperitoneally injected with Freund's incomplete adjuvant (Sigma). The anti-rTgGRA23 mouse sera were collected 10 days after the last immunization. Serum against rTgGRA7 and rTgGRA23 were induced in rabbits as follows: five hundred micrograms of rTgGRA7 or rTgGRA23 in Freund's complete adjuvant were intradermally injected into a female Japanese white rabbit (CLEA Japan) (day 0). Three hundred micrograms of rTgGRA7 or rTgGRA23 in Freund's incomplete adjuvant were intradermally injected into the rabbit on days 14 and 28. The anti-rTgGRA7 and anti-rTgGRA23 sera were collected from each rabbit 10 days after the last immunization. The specificity of the anti-rTgGRA7 rabbit serum was confirmed by immunoblotting (Supplemental Fig. S1). All mice and rabbits used in this study were treated under the guiding principles for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.6. Immunoblotting

Samples were dissolved in 2 × SDS-PAGE sample buffer and heated at 96 °C for 5 min after which they were separated by SDS-PAGE (12% gel). SDS-PAGE-separated proteins were electrically transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk diluted in PBS containing 0.05% Tween 20 (PBS-T) and probed with the following primary antibodies: anti-rTgGRA1 (1:1500), anti-rTgGRA23 rabbit antisera (1:1500), anti-TgGRA7 rabbit antiserum (1:2000), anti-TgSAG1 mouse monoclonal antibody (1:2000), or *T. gondii*-infected mouse antiserum (1:5000). The membranes were washed with PBS-T, and a secondary antibody that was either horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:4000) (Bethyl Laboratories, Montgomery, TX, USA) or HRP-conjugated anti-rabbit IgG antibody (1:4000) (Bethyl) was used to identify specific signals. Finally, bands were visualized using the Western lightning PLUS-ECL kit (PerkinElmer, Waltham, MA, USA) and the Versa Doc Imaging System (Bio Rad, Hercules, CA, USA).

2.7. Immuno-electron microscopic analysis

Confluent Vero cells (25-cm^2 flask) were infected with 5×10^6 *T. gondii* RH tachyzoites. Three days after infection, the cells were scraped from the flask and washed three times with PBS. Cells were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde in PBS for 2 h at 4 °C and then washed thoroughly in PBS. After dehydration with an ethanol series (50%: 0 °C for 15 min; 60%: 0 °C for 15 min;