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Host cell binding of GRA10, a novel, constitutively secreted dense granular protein from *Toxoplasma gondii*

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Abstract

Monoclonal antibodies (mAbs) against *Toxoplasma gondii*, Tg378 and Tg556 clones, are specifically observed to localize to the dense granules of tachyzoites by immunofluorescence microscopy. mAb Tg556 is directed against GRA3, a previously described 30 kDa dense granular protein. mAb Tg378 is directed against a novel 36 kDa dense granular protein, which we refer to as GRA10. These are major proteins in the excretory/secretory proteins from *T. gondii* before the parasite's entry into host cells, and they are released into the parasitophorous vacuole (PV) during or shortly after invasion to be associated with the PV membrane. GRA10 binds to the membrane of the host cells regardless of its anchorage-dependence or -independence. The cDNA sequence encoding GRA10 was determined by screening a *T. gondii* cDNA expression library with mAb Tg378. The deduced amino acid sequence of GRA10 consists of a polypeptide of 364 amino acids, and it has no significant homology to any other known proteins. The sequence contains amino terminal signal peptides and two potential transmembrane domains in the middle of sequence that are not near the carboxy terminus. GRA10 has a RGD motif between the two potential transmembrane domains.

Keywords: *Toxoplasma gondii*; Excretory/secretory proteins; GRA10; Host cell binding; Transmembrane domain; RGD motif

Toxoplasma gondii is an obligate intracellular protozoan parasite and an important zoonotic pathogen that is capable of invading and replicating within a wide variety of nucleated host cells. *T. gondii* causes severe diseases after congenital infection and in such immunocompromised patients as AIDS victims [1,2], in addition to the parasite infecting healthy persons [3].

Three secretory organelles are present in the *T. gondii* cytoplasm: the micronemes and the rhoptries discharge their contents from the apical end of the parasite and the dense granules discharge from the apical, lateral, and posterior surfaces. Until now, the contents of the three secretory organelles have been known to be released sequentially according to a cascade mode [4–6]. The microneme proteins (MIC1–MIC11) are released first, upon contact with the host cells, and they are thought to function for host cell recognition and attachment [7–9]. The contents of the rhoptries (ROP1–ROP9) are released next, and they may function in the formation of the parasitophorous vacuole (PV) [10–13]. The dense granular proteins (GRA1–GRA9) are exocytosed both during and after invasion into the PV. The exocytosed dense granular proteins either remain soluble in the lumen of the PV or they become associated with the PV membrane or the tubulo-reticular network of membranes within the PV [14]. The dense granule proteins are thought to modify the environment within the PV, thereby functioning for intracellular survival and replication [15]. However, GRA proteins are released constitutively in a calcium-independent fashion before the entry into the host cells [16] and moreover, they are the major portion of *T. gondii* excretory/

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secretory proteins (ESP [17]). This implies the concerted secretion of the dense granular proteins with secretions of the micronemal and rhoptry proteins.

During the screening of a *T. gondii* cDNA expression library with monoclonal antibodies [17–20], an expressed cDNA clone was detected by a mAb (Tg378) that blotted a dense granular 36 kDa protein. In this report, we describe the use of mAbs to identify and characterize a novel dense granular protein of *T. gondii*, GRA10, which functions during the entry of *T. gondii* into the host cells and also during the growth of the parasite within the host cells.

Materials and methods

Parasite and preparation of ESP. The RH strain of *T. gondii* was maintained via peritoneal passage in Balb/c mice. Prior to their use, the tachyzoites were purified by centrifugation over 40% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) in PBS solution [20]. The purified tachyzoites (3×10^8) were incubated at 37 °C for 1 h under mild agitation in 1.0 ml of Hanks' balanced salt solution (Gibco BRL, Rockville, MD). After centrifugation for 5 min at 6000 rpm, the supernatant was saved as the ESP [17].

Western blotting. The proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and Western blotting as described previously [21]. The nitrocellulose sheets blocked with 5% skim milk in PBS/0.05% Tween 20 (PBS/T) were incubated with 1:1000 diluted mAb, and then with 1:5000 diluted HRP-conjugated goat anti-mouse IgG antibody (Sigma Chem, St. Louis, MO). They were soaked in enhanced chemiluminescence (ECL) solution (Amersham Pharmacia Biotech) for 1 min and then exposed to an X-ray film (Konica, Tokyo, Japan).

Immunofluorescence assay on the free tachyzoites and on the invaded tachyzoites. Free tachyzoites were attached to 18-mm coverslips via a cytospin. The tachyzoites were fixed with cold absolute methanol for 5 min. Vero cells (CRL 6318, American Type Culture Collection, Rockville, MD) were maintained in DMEM supplemented with 10% FBS (Gibco BRL). These cells cultured on 18-mm coverslips in 24-well plates were infected with tachyzoites. The cells were then fixed either with cold absolute methanol for 5 min or with 3% formaldehyde for 10 min and then permeabilized by 0.05%(v/v) Triton X-100 for 5 min. MAb was diluted 1:200 in 3% BSA/PBS, and the FITC-conjugated goat anti-mouse IgG antibody (Sigma Chem) was used at a dilution of 1:500. The fluorescence was observed under a fluorescence microscope (Axiophot, Carl Zeiss, Oberkochen, Germany).

Cell culture and host cell binding assay. Vero76 (CRL 1587, ATCC), NIH/3T3 (CRL 1658), A549 (CCL 185), and SP2/0 (CRL 1581) cells were maintained in DMEM supplemented with 10% FBS (Gibco BRL). For the binding assay, Vero76, NIH/3T3, and A549 cells were incubated with 300 μl ESP for 30 min in 35-mm dishes. After washing with PBS, the cells were harvested with SDS-sample buffer. Western blot was performed with mAbs Tg378 and Tg556. And the SP2/0 cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Fab fraction, Sigma Chem.) for 30 min on ice after washing with PBS/1% BSA. The cells were fixed with 1% paraformaldehyde in PBS to check the fluorescence in a flow cytometry (BRYTE HS, Bio-Rad, Hercules, CA). Shifting of the fluorescence was analyzed with the winMDI program to the percentage of control without mAbs.

cDNA library screening. A *T. gondii* λZAPII cDNA expression library was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (McKesson Biosciences, Rockville, MD) and screened in *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, CA) using mAb in PBS/T containing 1%(w/v) BSA. Bound antibodies were detected using the ECL Detection System (Amersham Pharmacia Biotech). The positive plaques were recovered and rescreened by the same procedure. pBluescript SK phagemids were isolated by co-infection of the λZAPII phage and the ExAssist helper phage (Stratagene). The excised phagemids were further propagated in the *E. coli* SOLR host strain (Stratagene). Phagemid DNA was purified from single colonies using the Wizard Plus SV Miniprep kit (Promega, Madison, WI).

DNA sequencing, analysis of DNA, and the protein sequences. All DNA sequencing was performed using a dye terminator fluorescent-based sequence analysis on an Applied Biosystems 373 automated sequencer. The ends of the cDNA clones were sequenced using primers directed against the vector T7 and T3 promoter sequences. All the other sequencing primers were custom synthesized (Bionia, Daejon, Korea). Sequences of the cDNA clones were used to search for homologous sequences in the *Toxoplasma* dbEST (Database of Expressed Sequence Tags) using the BLASTn algorithm with default settings. The protein sequences were compared to the GenBank database using BLASTp. PROSITE was used to search for motifs and for post-translational modification sites. Hydropathicity of the amino acid sequence was obtained from ExPASy using the Kyte and Doolittle calculation [22].

5′ Rapid amplification of cDNA ends. The total *T. gondii* tachyzoite RNA was extracted using Tri reagent (Sigma Chem) according to the manufacturer's instructions. The 5′ untranslated region was amplified using the 5′ rapid amplification of cDNA ends (5′-RACE) procedure [23]. The first strand cDNA was synthesized from 1 μg of total RNA by using the Superscript Preamplification System (Life Technologies, Gaithersburg, MD). DeoxyCTPs were added to the 3′ end of the non-coding cDNA using terminal deoxynucleotide transferase (Life Technologies). PCR amplification of C-tailed cDNA was performed with an anchor primer (5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3′; Bionia) and a gene-specific primer (5′-CTT CCG CAG GTT CCC CTA GCT CTG-3′). The first-round product was further amplified with the abridged universal anchor primer (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′) and an internal gene-specific primer (5′-CTC TGC GTG GCT TTT CTC ACC AGC-3′). The second-round PCR product was cloned into the pGEM-T EASY vector (Promega), and it was sequenced using primers against the T7 and T3 vector promoter sequences.

Triton X-114 phase partitioning. Tachyzoites were extracted at 1×10^9 parasites per ml in extraction buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl), 0.5%(v/v) precondensed Triton X-114 (Pierce, Rockford, IL), and 1:100(v/v) dilution of aqueous and DMSO protease inhibitor stocks for 90 min on ice). The extract was centrifuged twice at 13,000*g* for 5 min at 4 °C. The cleared extract (500 μl) was overlaid on a 750 μl cushion of 6%(w/v) sucrose, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05%(v/v) precondensed Triton X-114, incubated for 5 min at 37 °C, and then centrifuged to separate the detergent and aqueous phases [24]. The phase partitioning was repeated once for each phase.

Production of recombinant GST fusion protein. RT-PCR was performed to amplify the DNA fragments of the open reading frame of the protein with a forward primer of 5′-CCG GAA TTC GGC ATG ATT GAG GCC GCT GTG-3′ and a reverse primer of 5′-ATA AGA ATG CGG CCG CTA AAC TAT TCA GAC AGG CGT TTC CCC-3′ that were directed against the sequence. The amplified DNA was digested with *Eco*RI/*Not*I for inserting it into the pGEX-4T-1 plasmid (Pharmacia Biotech, Sweden). The DNA was used to transform the host *E. coli* (JM105). The expression of GST fusion protein was induced with 0.1 mM IPTG for 3 h at 30 °C.

Results

ESP from the free tachyzoites was well prepared without any reaction with the major surface protein (SAG1) blotted by mAb Tg563 (Fig. 1). The mAbs Tg378 and