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Characterization of a novel thrombospondin-related protein in *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is an obligate intracellular protozoan parasite that invades a wide range of host cells. Upon encountering host cells, the parasite releases a large variety of proteins from secretory organelles, such as micronemes, rhoptries and dense granules. The secretion of microneme protein is essential for parasite invasion. We found that a secreted protein with an altered thrombospondin repeat of Toxoplasma gondii (TgSPATR) was a novel microneme protein, which was different from known microneme proteins that carry thrombospondin repeat domains. TgSPATR was secreted in response to an intra-parasitic elevation of Ca\textsuperscript{2+} and probably secreted during early stages of parasite invasion. Thus, we suggested that TgSPATR, new member of microneme secretory protein, had a possible function in the invasion.

Keywords: Ca\textsuperscript{2+}-dependent secretion, microneme protein, secreted protein with an altered thrombospondin repeat, Toxoplasma gondii
The intracellular protozoan parasite, *Toxoplasma gondii*, is a pathogen of the zoonosis, toxoplasmosis. It causes severe opportunistic disease in congenitally infected babies and immunocompromised individuals (i.e. AIDS patients) [1,2]. Infection in livestock is a threat to public health from food-borne outbreaks and causes great economic loss [3]. Thus, accurate surveillance and effective precaution against this infectious disease is strongly sought after. Research into the key molecules of *T. gondii* will contribute to the development of diagnostic techniques and aid clinical treatment.

*T. gondii* belongs to the phylum Apicomplexa, which includes other pathogens of medical and veterinary importance such as *Plasmodium, Cryptosporidium, Sarcocystis, Eimeria*, and *Neospora*. These protozoan parasites are characterized by the presence of a peculiar organelle complex at their apical end [4]. The complex includes specialized secretory organelles, namely micronemes, rhoptries, and dense granules [5]. Previous studies have revealed that proteins, sequentially secreted from these organelles, play important roles in parasite invasion and the establishment of infection [6,7]. Adhesins, a group of proteins carrying adhesive properties, are secreted from micronemes at the moment of invasion, and the secretion is essential for parasite-host cell interaction [8].

Micronemes of apicomplexa parasites contain a conserved family of proteins that serves
as adhesins [8]. In *T. gondii*, one of the microneme secretory proteins, MIC2, belongs to the thrombospondin-related anonymous protein (TRAP) family, which is conserved through the phylum [9]. The conditional knockout of *mic2* gene causes a significant reduction in infectivity [10]. The deletion of the microneme protein 2-associating protein, M2AP, which escorts MIC2 throughout the secretion, also strongly suppresses invasion [11]. Another microneme secretory protein, apical membrane antigen 1 (AMA1), is common in the phylum, and *ama1* gene knockout in *T. gondii* impairs the ability to invade host cells [12,13]. Thus, secretory proteins in micronemes are indispensable for parasite invasion, and are potential candidates as vaccine and drug targets [10,14].

Microneme secretion is regulated by intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of the parasite [15]. Ca\(^{2+}\) ionophores (A23187 and ionomycin) and other Ca\(^{2+}\)-mobilizing agents (Thapsigargin, NH\(_4\)Cl, caffeine, ryanodine and ethanol) increase [Ca\(^{2+}\)]\(_i\) in *T. gondii* and induce subsequent microneme secretion [15-17]. A phytohormone, abscisic acid, causes MIC2 secretion via the production of cyclic ADP ribose, and is probably a physiological [Ca\(^{2+}\)]\(_i\) modulator in *T. gondii* [18]. In contrast, reduction in [Ca\(^{2+}\)]\(_i\) by a cell-permeable Ca\(^{2+}\) chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), strongly suppresses secretion and parasite invasion [15].

Ca\(^{2+}\)-mobilizing agents provide a convenient way to artificially induce microneme
secretion. We had performed a proteomic analysis of Ca$^{2+}$ ionophore-dependent secretion, and found candidates for novel microneme proteins in *T. gondii* [19] (Kawase et al. 2007). One of them was a homolog of the secreted protein with an altered thrombospondin repeat (SPATR) of the *Plasmodium* species and it was named TgSPATR. *P. falciparum* SPATR (PfSPATR) carries adhesive domains, namely epidermal growth factor (EGF)-like domain and a thrombospondin type I repeat (TSR) domain, and is localized on the surface of sporozoites and around the rhoptries in asexual erythrocytic stages [20,21]. Additionally, anti-serum against PfSPATR suppresses sporozoite invasion, suggesting that it works as an adhesin in *P. falciparum* [20].

Here, we showed that TgSPATR is definitely classified into the SPATR family after comparison with MIC2 and TRAPs, which also have TSR domains. This is the first study that the *spatr* gene is cloned from a genus other than *Plasmodium*. Furthermore, we showed TgSPATR was localized in microneme, and secreted in a Ca$^{2+}$-dependent manner, probably during the early stages of parasite invasion. Thus, TgSPATR is a novel microneme secretory protein and may be involved in parasite invasion. Our findings indicate that SPATR is conserved not only in *Plasmodium* species, but also in *Toxoplasma gondii*, suggesting that SPATRs are significant for the survival of apicomplexan parasites.
Materials and methods

Parasite culture and preparation

Vero cells were grown in Minimum Essential Medium Eagle (Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin. The *T. gondii* strain RH was maintained in a monolayer of Vero cells. Parasites were purified by sequential passages of infected Vero cells through 25- and 27-gauge needles and a 5 μm pore size filter (Millex®-SV, from Millipore, Billerica, MA). For immuno-electron microscopic analysis, CHO-K1 cells were used as host cells, and were grown in RPMI1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cloning of *tgspatr* gene and expression of recombinant TgSPATR

Parasite cDNA was synthesized from total parasite RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Inc., Carlsbad, CA) and was used as template DNA to amplify the complete and partial sequence of TgSPATR. A set of oligonucleotide primers, the forward primer (5’-CCATGGAGGTTTCAAGAAGTCACCGGT-3’) and reverse primer (5’-ATCCCGGGTTAAGACGAAGGCTGATTGCA-3’), was used to amplify the full-length
sequence, composed of 534 amino acids. The PCR product was ligated into a TA-cloning vector, pCR 2.1-TOPO, using the TOPO TA Cloning Kit (Invitrogen Inc.). A partial sequence (amino acids 95–433) was amplified using the other primers, the forward primer (5′-ATGAATTCCCCTCGGATGCCGCTGGCGAC-3′) and reverse primer (5′-ATGCGGCCGCTCAGAGCTCGTAGATGAAGTCGAC-3′), and ligated into the glutathione S-transferase (GST)-fused *Escherichia coli* expression vector pGEX-4T1 (GE Healthcare, Buckinghamshire, England) after digesting the PCR product and vector with *Eco*RI and *Not*I. The nucleotide sequences were analyzed with model ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA). A partial TgSPATR (pTgSPATR) was expressed as GST-fusion protein in *E. coli* DH5α strain, and then purified according to the manufacturer’s instructions (GE Healthcare).

### Phylogenetic analysis

A phylogenetic tree was produced using sequence analysis software, CLC Free Workbench. Neighbor joining method with bootstrap analysis (100 replicates) was performed to construct the tree. The sequences of MIC2, TRAPs, and SPATRs of *Toxoplasma gondii* (Tg), *Plasmodium falciparum* (Pf), *P. vivax* (Pv), *P. knowlesi* (Pk), *Neospora caninum* (Nc), and *Babesia gibsoni* (Bg) were obtained from the NCBI database (ID number of TgMIC2: **AAB63303**, PfTRAP: **AAA29776**, PfSPATR: ****).
XP_001349632, PvTRAP: AAC97485, PvSPATR: AAX53168, PkTRAP: AAG24613, PkSPATR: AAX51302, NcTRAP: AAF01565, BgTRAP: BAB68553), and their TSR domains were determined by SMART (http://smart.embl-heidelberg.de/) or ScanProsite (http://www.expasy.org/tools/scanprosite/), which are the servers for sequence pattern and profile searches. Because more than one TSR domain was found in TgMIC2 and NcTRAP, each domain was numbered and analyzed as an individual TSR sequence.

Production of anti-TgSPATR serum

To produce anti-serum in mice, 100 μg of pTgSPATR in Freund’s complete adjuvant (Sigma) was intraperitoneally injected into ICR mice (6-week-old, female; CLEA Japan Inc., Tokyo, Japan), followed by intraperitoneal injections of 50 μg pTgSPATR in Freund’s incomplete adjuvant (Sigma) on days 14, 28, and 42. For rabbit anti-serum, 1 mg of pTgSPATR in Freund’s complete adjuvant (Sigma) was subcutaneously injected into Japanese white rabbits (12-week-old, female; CLEA Japan Inc.), followed by subcutaneous injections of 500 μg pTgSPATR in Freund’s incomplete adjuvant (Sigma) on days 14, 28, and 42. Serum was collected from mice or rabbits on 13 days after the last immunization. Animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals Promulgated by the Obihiro University of Agriculture and
Indirect fluorescent antibody test (IFAT)

Parasites were cultured on a cell sheet of Vero cells, attached to coverslips. The coverslips were washed three times with PBS, and then fixed with PBS containing 3% paraformaldehyde (PFA). They were treated with 0.5% saponin in PBS for 15 min, to permeabilize the plasma membrane of both Vero cells and parasites. After washing three times with PBS containing 0.002% saponin (PBSS), the coverslips were blocked with 3% bovine serum albumin (BSA) in PBSS for 1 h. The primary antibody, rabbit anti-TgSPATR serum, mouse anti-TgSPATR serum, or mouse anti-MIC2 antibody (clone ID: T3-4A11), was diluted 1:200 in PBSS, and the secondary antibody, Alexa488-conjugated goat anti-rabbit IgG (Sigma) or Alexa594-conjugated goat anti-mouse IgG antibody (Sigma), was diluted 1:200 in PBSS. To see TgSPATR exposed or secreted outside the parasite, the plasma membrane of the Vero cells, but not the parasites, was permeabilized by replacing 0.5% saponin with 0.002% saponin [6].

Induction of microneme secretion and detection of secreted proteins

Parasites were suspended in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose,
and 50 mM HEPES pH 7.4) supplemented with 1 mM CaCl₂, and were adjusted to 1 × 10⁸ cells/ml.

To induce secretion, 1% ethanol or 1% DMSO was added to the parasites, and then incubated at 37°C for 10 min. To inhibit secretion (Wako Pure Chemical Industries, Osaka, Japan), parasites were pre-treated with 50 μM BAPTA-AM at 18°C for 20 min (Nagamune et al. 2008). The supernatant was collected by sequential centrifugations (500 × g, 10 min, 20°C and then 10,000 × g, 10 min, 20°C), and applied to SDS-PAGE with 10% polyacrylamide gel, followed by western blot analysis.

To detect TgSPATR, *Toxoplasma* β-actin (Tgβ-actin), and MIC2, each primary antibody, mouse anti-TgSPATR serum, mouse anti-Tgβ-actin serum, or mouse anti-MIC2 monoclonal antibody, was diluted 1:500 in PBS containing 0.05% Tween20 (PBST), and the secondary antibody, horseradish peroxidise (HRP)-conjugated goat anti-mouse IgG antibody (Benthyl Inc., Montgomery, TX), was diluted 1:10,000 in PBST.

**Immuno-electron microscopic (IEM) analysis**

CHO-K1 cells, infected with the parasites, were scraped and washed three times with PBS. Cells were fixed in 4% PFA including 0.1% glutaraldehyde and 8% sucrose in 0.1M phosphate buffer (pH 7.4) overnight at 4°C, washed in 0.1M phosphate buffer (pH 7.4) and embedded in 2% agarose. After dehydration with an ethanol series, the samples were embedded in LR Gold resin (Polysciences Inc.,
Warrington, PA). Thin sections (about 80 nm thick) were cut on a Leica UCT ultramicrotome using a diamond knife and placed on nickel grids. Sections were exposed at room temperature for 30 min to 5% skim milk in PBS as a blocking agent, then treated with mouse anti-TgSPATR serum overnight at 4°C and subsequently treated with 10 nm gold-labeled goat anti-mouse IgG antibody (GE Healthcare) at room temperature for 2 h. Normal mouse serum was used instead of the primary antibody as a negative control. These sections were counter-stained with uranylacetate before examination with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

Results

Cloning and sequence analysis of TgSPATR

The *tgspatr* gene was cloned from *T. gondii* cDNA and the sequence corresponded to that of a hypothetical protein (83.m00006), listed in the *Toxoplasma* database (ToxoDB, http://toxodb.org/toxo/). We found that TgSPATR contained a TSR domain, and SPATRs of *Plasmodium* species were the most homologous proteins in the NCBI protein database (http://www.ncbi.nlm.nih.gov). As described in previous paper, N-terminal sequence of secreted TgSPATR was determined as ESPXD, which was a cleavage site other than putative signal sequence
It suggested that this protein is cleaved during secretion steps, similarly to known microneme proteins [22]. Furthermore, the phylogenetic tree of TSR domains clearly discriminated SPATRs from the other proteins, which possess TSR domains (Fig. 1b). These results indicated that TgSPATR certainly belonged to the SPATR family.

**Localization of TgSPATR in micronemes**

We investigated the cellular localization of TgSPATR, by using immunofluorescence double staining and IEM analysis. The fluorescent images showed that TgSPATR was co-localized with MIC2 in the apical end of *T. gondii* (Fig. 2a-d). Additionally, IEM analysis demonstrated that TgSPATR was localized in the slightly dense structures of the parasite apical end, which must be micronemes because they were clearly distinguished from rhoptries and looked quite similar to micronemes shown in previous reports (Fig. 2e) [12,15]. From these results, we concluded that TgSPATR was a microneme protein.

**Calcium-dependent secretion of TgSPATR**

Ethanol is one of the most effective agents for inducing $[\text{Ca}^{2+}]$ elevation and subsequent $\text{Ca}^{2+}$-dependent secretion in *T. gondii* [16]. We confirmed that ethanol elevated $[\text{Ca}^{2+}]$, and increased
the amount of protein released from the parasites, previously [19]. Here, the secretion of TgSPATR, induced by ethanol, was clearly demonstrated by western blotting (Fig. 3). The suppression of ethanol-induced secretion by a cell-permeable Ca\(^{2+}\) chelator, BAPTA-AM, strongly supported Ca\(^{2+}\)-dependency. These changes corresponded to those of MIC2, certifying the accuracy of our previous experiment, and suggested that TgSPATR was secreted in a Ca\(^{2+}\)-dependent manner [19].

**Spatiotemporal analysis of TgSPATR secretion**

A high concentration of saponin (0.5%) permeabilizes the plasma membranes of both host cells and intracellular parasites, while a low concentration of saponin (0.002%) permeabilizes the plasma membrane of host cells, but not that of parasites [6]. Thus, 0.5% saponin makes all parasite proteins detectable in IFAT, because of the complete permeabilization of plasma membranes of host cells and parasites. Conversely, in 0.002% saponin-permeabilized cells, the proteins exposed or secreted outside the parasite, are only detected by IFAT, because the parasite membrane is not permeabilized and the antibody does not reach the intra-parasite proteins.

We performed IFAT in infected host cells to estimate when and where TgSPATR was secreted from the parasites. In the case of 0.5% saponin, TgSPATR was detected around the apical end of both extracellular and intracellular parasites (Fig. 4a, lower two images). However, in the case
of 0.002% saponin, TgSPATR disappeared in intracellular parasites (Fig. 4a, upper two images). The images suggest that TgSPATR was secreted and shed from the surface of parasite during the early events of invasion. Furthermore, MIC2, which is a secretory microneme protein and lost from the parasite surface during invasion, was detected in similar manner to TgSPATR (Fig. 4b). Thus, we succeeded to selectively permeabilized the plasma membrane of host cell and TgSPATR was probably secreted during the early events of invasion.

Taking all result into consideration, we concluded that TgSPATR was new microneme protein, Ca\(^{2+}\)-dependently secreted during early events of parasite invasion.

Discussion

This is the first report of the spatr gene, cloned from a species other than Plasmodium. Sequence analysis of SPATRs, TRAPs and MIC2 clearly showed that TgSPATR belonged to SPATR family (Fig. 1). We suggested that SPATR, or the proto-SPATR, existed before the birth of genera Toxoplasma and Plasmodium, because the TSR domains of SPATRs were clustered in adjacent branches of the phylogenetic tree (Fig. 1a). The old origin of SPATRs may imply their important roles in parasite survival.

IFAT and IEM analysis showed TgSPATR was localized in microneme. Additionaly,
Ca\textsuperscript{2+}-dependent secretion of TgSPATR was confirmed using ethanol and an intracellular Ca\textsuperscript{2+} chelator. Major microneme proteins, such as MIC2, 4, 10, 11 and AMA1, are secreted in Ca\textsuperscript{2+}-dependent manner, suggesting TgSPATR is a typical microneme protein [12,15,23,24]. Interestingly, the localization of TgSPATR was quite different from PfSPATR, which was localized on the surface of sporozoites and around the rhoptries in asexual erythrocytic stages, but not in any intracellular organelles [20]. TgSPATR is likely to behave and function in a different way from PfSPATRs, because the putative molecular sizes of TgSPATR and PfSPATR are much different (58 kDa and 30 kDa, respectively). The specific region of TgSPATR may be a key for targeting micronemes. Two conserved motifs (SYHYY and EIEYE) in the cytoplasmic domain of transmembrane-type MICs are necessary for targeting micronemes, and the propeptide and EGF domains of a soluble protein, MIC3, are important for its localization in micronemes [25,26]. But, TgSPATR does not have such sorting signals, and might be transported into micronemes by a unique recognition mechanism.

We succeeded to selectively detect TgSPATR exposed outside parasites, and suggested that TgSPATR was secreted during the early events of invasion. This result suggested a possible function of TgSPATR in the parasite invasion. However, its actual function remains unclear because the anti-TgSPATR antibody used in this research does not inhibit parasite invasion (data not shown). The lack of inhibitory effect may be due to the redundancy of a similar protein, for example MIC2.
The amount of MIC2 in A23187-induced secretion is much higher than that of TgSPATR (about 11-fold), according to our previous research [19]. Or, TgSPATR may be an escorter that guides other proteins to micronemes and/or parasite surface, like MIC6 and M2AP [11,27]. In this case, the antibody does not inhibit parasite invasion, because TgSPATR has no direct function in invasion. Thus, additional experiments, particularly investigation of over-expression and deletion of the gene, are required to clarify the function of TgSPATR.

In this research, we concluded that TgSPATR was a new microneme secretory protein. It is probably secreted during early events of parasite invasion, suggesting a putative function in the invasion. Although the exact function remains unclear, we believe that future experiments will reveal the unique nature of TgSPATR and its potential as vaccine and drug target.

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References


[15] Carruthers VB, Sibley LD. Mobilization of intracellular calcium stimulates microneme


Figure legends

Fig. 1. Sequence analysis of TgMIC2, TRAPs, and SPATRs. a) Amino acid sequence of TgSPATR included putative signal sequence (white rectangle), cleaved site (gray rectangle) and TSR domain (under bar). TgSPATR has a WSXW motif (black rectangle), which is conserved in SPATRs of Plasmodium species. b) The branches, enclosed with a rectangle, include all SPATRs. In case of TgMIC2 and NcTRAP, each TSR domain is numbered from N-terminal side and the position is indicated in parentheses. Species are indicated as Tg: *Toxoplasma gondii*, Pf: *Plasmodium falciparum*, Pv: *P. vivax*, Pk: *P. knowlesi*, Nc: *Neospora caninum*, and Bg: *Bagesia gibsoni*.

Fig. 2. Localization of TgSPATR in micronemes. Extracellular *T. gondii* was stained using rabbit anti-TgSPATR serum and subsequent Alexa488-labeled secondary antibody (green, b), or using mouse anti-MIC2 monoclonal antibody and Alexa594-labeled secondary antibody (Red, c). Signals of TgSPATR and MIC2 were merged in d. The phase-contrast image corresponds to the fluorescence images (a). In the IEF image of intracellular parasites, arrows indicate signals in slightly dense structures of the parasite apical end (e). These structures are probably micronemes, not rhoptries (R).
Fig. 3. Calcium-dependent secretion of TgSPATR. TgSPATR, MIC2, and β-actin, secreted from parasites, were detected by using western blot analysis. Parasites were pretreated with 50 μM BAPTA-AM (+) or not (–), and then treated with 1% ethanol (+) or not (–). MIC2 was a positive control of Ca^{2+}-dependent secretion, while β-actin was a negative control, indicating the degree of parasite destruction.

Fig. 4. Spatiotemporal analysis of TgSPATR secretion. Intracellular and extracellular parasites were fixed and localization of TgSPATR (a) and MIC2 (b) were analyzed by IFAT, using mouse anti-TgSPATR serum and mouse anti-MIC2 monoclonal antibody respectively. The preparations were permeabilized by 0.002% saponin to see the existence of TgSPATR and MIC2 on the outer surface of parasite. Or, they were permeabilized by 0.5% saponin to see the distribution of the proteins in both outside and inside parasite. The phase-contrast and fluorescence images are merged and arrows indicate the intracellular parasites lacking in TgSPATR or MIC2 on outer surface of parasites.