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

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14

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^{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^{2+}-dependent secretion, microneme protein, secreted protein with an altered thrombospondin repeat, *Toxoplasma gondii*

29 **Introduction**

30 The intracellular protozoan parasite, *Toxoplasma gondii*, is a pathogen of the zoonosis, toxoplasmosis.
31 It causes severe opportunistic disease in congenitally infected babies and immunocompromised
32 individuals (i.e. AIDS patients) [1,2]. Infection in livestock is a threat to public health from
33 food-borne outbreaks and causes great economic loss [3]. Thus, accurate surveillance and effective
34 precaution against this infectious disease is strongly sought after. Research into the key molecules of
35 *T. gondii* will contribute to the development of diagnostic techniques and aid clinical treatment.

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

45 Micronemes of apicomplexa parasites contain a conserved family of proteins that serves

46 as adhesins [8]. In *T. gondii*, one of the microneme secretory proteins, MIC2, belongs to the

47 thrombospondin-related anonymous protein (TRAP) family, which is conserved through the phylum

48 [9]. The conditional knockout of *mic2* gene causes a significant reduction in infectivity [10]. The

49 deletion of the microneme protein 2-associating protein, M2AP, which escorts MIC2 throughout the

50 secretion, also strongly suppresses invasion [11]. Another microneme secretory protein, apical

51 membrane antigen 1 (AMA1), is common in the phylum, and *ama1* gene knockout in *T. gondii*

52 impairs the ability to invade host cells [12,13]. Thus, secretory proteins in micronemes are

53 indispensable for parasite invasion, and are potential candidates as vaccine and drug targets [10,14].

54 Microneme secretion is regulated by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of the

55 parasite [15]. Ca^{2+} ionophores (A23187 and ionomycin) and other Ca^{2+}-mobilizing agents

56 (Thapsigargin, NH_4Cl, caffeine, ryanodine and ethanol) increase $[Ca^{2+}]_i$ in *T. gondii* and induce

57 subsequent microneme secretion [15-17]. A phytohormone, abscisic acid, causes MIC2 secretion via

58 the production of cyclic ADP ribose, and is probably a physiological $[Ca^{2+}]_i$ modulator in *T. gondii*

59 [18]. In contrast, reduction in $[Ca^{2+}]_i$ by a cell-permeable Ca^{2+} chelator,

60 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM),

61 strongly suppresses secretion and parasite invasion [15].

62 Ca^{2+}-mobilizing agents provide a convenient way to artificially induce microneme

63 secretion. We had performed a proteomic analysis of Ca^{2+} ionophore-dependent secretion, and found

64 candidates for novel microneme proteins in *T. gondii* [19] (Kawase *et al.* 2007). One of them was a

65 homolog of the secreted protein with an altered thrombospondin repeat (SPATR) of the *Plasmodium*

66 species and it was named TgSPATR. *P. falciparum* SPATR (PfSPATR) carries adhesive domains,

67 namely epidermal growth factor (EGF)-like domain and a thrombospondin type I repeat (TSR)

68 domain, and is localized on the surface of sporozoites and around the rhoptries in asexual

69 erythrocytic stages [20,21]. Additionally, anti-serum against PfSPATR suppresses sporozoite invasion,

70 suggesting that it works as an adhesin in *P. falciparum* [20].

71 Here, we showed that TgSPATR is definitely classified into the SPATR family after

72 comparison with MIC2 and TRAPs, which also have TSR domains. This is the first study that the

73 *spatr* gene is cloned from a genus other than *Plasmodium*. Furthermore, we showed TgSPATR was

74 localized in microneme, and secreted in a Ca^{2+}-dependent manner, probably during the early stages of

75 parasite invasion. Thus, TgSPATR is a novel microneme secretory protein and may be involved in

76 parasite invasion. Our findings indicate that SPATR is conserved not only in *Plasmodium* species, but

77 also in *Toxoplasma gondii*, suggesting that SPATRs are significant for the survival of apicomplexan

78 parasites.

79

80 **Materials and methods**

81

82 **Parasite culture and preparation**

83 Vero cells were grown in Minimum Essential Medium Eagle (Sigma, St. Louis, MO) supplemented

84 with 8% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin. The *T. gondii* strain RH was

85 maintained in a monolayer of Vero cells. Parasites were purified by sequential passages of infected

86 Vero cells through 25- and 27-gauge needles and a 5 μm pore size filter (Millex®-SV, from Millipore,

87 Billerica, MA). For immuno-electron microscopic analysis, CHO-K1 cells were used as host cells,

88 and were grown in RPMI1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum,

89 100 U/ml penicillin and 100 μg/ml streptomycin.

90

91 **Cloning of tgspatr gene and expression of recombinant TgSPATR**

92 Parasite cDNA was synthesized from total parasite RNA using SuperScript^{TM} First-Strand Synthesis

93 System for RT-PCR (Invitrogen Inc., Carlsbad, CA) and was used as template DNA to amplify the

94 complete and partial sequence of TgSPATR. A set of oligonucleotide primers, the forward primer

95 (5'-CCATGGAGGTTTCAAGAAGTCACCGGT-3') and reverse primer

96 (5'-ATCCCGGGTTAAGACGAAGGCTGATTGCA-3'), was used to amplify the full-length

97 sequence, composed of 534 amino acids. The PCR product was ligated into a TA-cloning vector,

98 pCR 2.1-TOPO, using the TOPO TA Cloning Kit (Invitrogen Inc.). A partial sequence (amino acids

99 95–433) was amplified using the other primers, the forward primer

100 (5'-ATGAATTCCCCTCGGATGCCGCTGGCGAC-3') and reverse primer

101 (5'-ATGCGGCCGCTCAGAGCTCGTAGATGAAGTCGAC-3'), and ligated into the glutathione

102 *S*-transferase (GST)-fused *Escherichia coli* expression vector pGEX-4T1 (GE Healthcare,

103 Buckinghamshire, England) after digesting the PCR product and vector with *Eco*RI and *Not*I. The

104 nucleotide sequences were analyzed with model ABI 3100 DNA sequencer (Applied Biosystems,

105 Foster City, CA). A partial TgSPATR (pTgSPATR) was expressed as GST-fusion protein in *E. coli*

106 DH5α strain, and then purified according to the manufacturer's instructions (GE Healthcare).

107

108 **Phylogenetic analysis**

109 A phylogenetic tree was produced using sequence analysis software, CLC Free Workbench. Neighbor

110 joining method with bootstrap analysis (100 replicates) was performed to construct the tree. The

111 sequences of MIC2, TRAPs, and SPATRs of *Toxoplasma gondii* (Tg), *Plasmodium falciparum* (Pf), *P.*

112 *vivax* (Pv), *P. knowlesi* (Pk), *Neospora caninum* (Nc), and *Babesia gibsoni* (Bg) were obtained from

113 the NCBI database (ID number of TgMIC2: **AAB63303**, PfTRAP: **AAA29776**, PfSPATR:

114 **XP_001349632**, PvTRAP: **AAC97485**, PvSPATR: **AAX53168**, PkTRAP: **AAG24613**, PkSPATR:

115 **AAX51302**, NcTRAP: **AAF01565**, BgTRAP: **BAB68553**), and their TSR domains were determined

116 by SMART (http://smart.embl-heidelberg.de/) or ScanProsite

117 (http://www.expasy.org/tools/scanprosite/), which are the servers for sequence pattern and profile

118 searches. Because more than one TSR domain was found in TgMIC2 and NcTRAP, each domain was

119 numbered and analyzed as an individual TSR sequence.

120

121 **Production of anti-TgSPATR serum**

122 To produce anti-serum in mice, 100 µg of pTgSPATR in Freund's complete adjuvant (Sigma) was

123 intraperitoneally injected into ICR mice (6-week-old, female; CLEA Japan Inc., Tokyo, Japan),

124 followed by intraperitoneal injections of 50 µg pTgSPATR in Freund's incomplete adjuvant (Sigma)

125 on days 14, 28, and 42. For rabbit anti-serum, 1 mg of pTgSPATR in Freund's complete adjuvant

126 (Sigma) was subcutaneously injected into Japanese white rabbits (12-week-old, female; CLEA Japan

127 Inc.), followed by subcutaneous injections of 500 µg pTgSPATR in Freund's incomplete adjuvant

128 (Sigma) on days 14, 28, and 42. Serum was collected from mice or rabbits on 13 days after the last

129 immunization. Animals used in this study were cared for and used under the Guiding Principles for

130 the Care and Use of Research Animals Promulgated by the Obihiro University of Agriculture and

131 Veterinary Medicine.

132

133 **Indirect fluorescent antibody test (IFAT)**

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

145

146 **Induction of microneme secretion and detection of secreted proteins**

147 Parasites were suspended in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose,

148 and 50 mM HEPES pH 7.4) supplemented with 1 mM $CaCl_2$, and were adjusted to 1 × 10^8 cells/ml.

149 To induce secretion, 1% ethanol or 1% DMSO was added to the parasites, and then incubated at 37°C

150 for 10 min. To inhibit secretion (Wako Pure Chemical Industries, Osaka, Japan), parasites were

151 pre-treated with 50 μM BAPTA-AM at 18°C for 20 min (Nagamune *et al.* 2008). The supernatant

152 was collected by sequential centrifugations (500 × *g*, 10 min, 20°C and then 10,000 × *g*, 10 min,

153 20°C), and applied to SDS-PAGE with 10% polyacrylamide gel, followed by western blot analysis.

154 To detect TgSPATR, *Toxoplasma* β-actin (Tgβ-actin), and MIC2, each primary antibody, mouse

155 anti-TgSPATR serum, mouse anti-Tgβ-actin serum, or mouse anti-MIC2 monoclonal antibody, was

156 diluted 1:500 in PBS containing 0.05% Tween20 (PBST), and the secondary antibody, horseradish

157 peroxidise (HRP)-conjugated goat anti-mouse IgG antibody (Benthyl Inc., Montgomery, TX), was

158 diluted 1:10,000 in PBST.

159

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

161 CHO-K1 cells, infected with the parasites, were scraped and washed three times with PBS. Cells

162 were fixed in 4% PFA including 0.1% glutaraldehyde and 8% sucrose in 0.1M phosphate buffer (pH

163 7.4) overnight at 4°C, washed in 0.1M phosphate buffer (pH 7.4) and embedded in 2% agarose. After

164 dehydration with an ethanol series, the samples were embedded in LR Gold resin (Polysciences Inc.,

165 Warrington, PA). Thin sections (about 80 nm thick) were cut on a Leica UCT ultramicrotome using a

166 diamond knife and placed on nickel grids. Sections were exposed at room temperature for 30 min to

167 5% skim milk in PBS as a blocking agent, then treated with mouse anti-TgSPATR serum overnight at

168 4°C and subsequently treated with 10 nm gold-labeled goat anti-mouse IgG antibody (GE

169 Healthcare) at room temperature for 2 h. Normal mouse serum was used instead of the primary

170 antibody as a negative control. These sections were counter-stained with uranylacetate before

171 examination with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

172

173 **Results**

174

175 **Cloning and sequence analysis of TgSPATR**

176 The *tgspatr* gene was cloned from *T. gondii* cDNA and the sequence corresponded to that of a

177 hypothetical protein (83.m00006), listed in the *Toxoplasma* database (ToxoDB,

178 http://toxodb.org/toxo/). We found that TgSPATR contained a TSR domain, and SPATRs of

179 *Plasmodium* species were the most homologous proteins in the NCBI protein database

180 (http://www.ncbi.nlm.nih.gov). As described in previous paper, N-terminal sequence of secreted

181 TgSPATR was determined as ESPXD, which was a cleavage site other than putative signal sequence

182 (Fig. 1a) [19]. It suggested that this protein is cleaved during secretion steps, similarly to known

183 microneme proteins [22]. Furthermore, the phylogenetic tree of TSR domains clearly discriminated

184 SPATRs from the other proteins, which possess TSR domains (Fig. 1b). These results indicated that

185 TgSPATR certainly belonged to the SPATR family.

186

187 **Localization of TgSPATR in micronemes**

188 We investigated the cellular localization of TgSPATR, by using immunofluorescence double staining

189 and IEM analysis. The fluorescent images showed that TgSPATR was co-localized with MIC2 in the

190 apical end of *T. gondii* (Fig. 2a-d). Additionally, IEM analysis demonstrated that TgSPATR was

191 localized in the slightly dense structures of the parasite apical end, which must be micronemes

192 because they were clearly distinguished from rhoptries and looked quite similar to micronemes

193 shown in previous reports (Fig. 2e) [12,15]. From these results, we concluded that TgSPATR was a

194 microneme protein.

195

196 **Calcium-dependent secretion of TgSPATR**

197 Ethanol is one of the most effective agents for inducing $[Ca^{2+}]_i$ elevation and subsequent

198 Ca^{2+}-dependent secretion in *T. gondii* [16]. We confirmed that ethanol elevated $[Ca^{2+}]_i$ and increased

199 the amount of protein released from the parasites, previously [19]. Here, the secretion of TgSPATR,

200 induced by ethanol, was clearly demonstrated by western blotting (Fig. 3). The suppression of

201 ethanol-induced secretion by a cell-permeable Ca^{2+} chelator, BAPTA-AM, strongly supported

202 Ca^{2+}-dependency. These changes corresponded to those of MIC2, certifying the accuracy of our

203 previous experiment, and suggested that TgSPATR was secreted in a Ca^{2+}-dependent manner [19].

204

205 **Spatiotemporal analysis of TgSPATR secretion**

206 A high concentration of saponin (0.5%) permeabilizes the plasma membranes of both host cells and

207 intracellular parasites, while a low concentration of saponin (0.002%) permeabilizes the plasma

208 membrane of host cells, but not that of parasites [6]. Thus, 0.5% saponin makes all parasite proteins

209 detectable in IFAT, because of the complete permeabilization of plasma membranes of host cells and

210 parasites. Conversely, in 0.002% saponin-permeabilized cells, the proteins exposed or secreted

211 outside the parasite, are only detected by IFAT, because the parasite membrane is not permeabilized

212 and the antibody does not reach the intra-parasite proteins.

213 We performed IFAT in infected host cells to estimate when and where TgSPATR was

214 secreted from the parasites. In the case of 0.5% saponin, TgSPATR was detected around the apical

215 end of both extracellular and intracellular parasites (Fig. 4a, lower two images). However, in the case

216 of 0.002% saponin, TgSPATR disappeared in intracellular parasites (Fig. 4a, upper two images). The

217 images suggest that TgSPATR was secreted and shed from the surface of parasite during the early

218 events of invasion. Furthermore, MIC2, which is a secretory microneme protein and lost from the

219 parasite surface during invasion, was detected in similar manner to TgSPATR (Fig. 4b). Thus, we

220 succeeded to selectively permeabilized the plasma membrane of host cell and TgSPATR was

221 probably secreted during the early events of invasion.

222 Taking all result into consideration, we concluded that TgSPATR was new microneme

223 protein, Ca^{2+}-dependently secreted during early events of parasite invasion.

224

225 **Discussion**

226 This is the first report of the *spatr* gene, cloned from a species other than *Plasmodium*. Sequence

227 analysis of SPATRs, TRAPs and MIC2 clearly showed that TgSPATR belonged to SPATR family

228 (Fig. 1). We suggested that SPATR, or the proto-SPATR, existed before the birth of genera

229 *Toxoplasma* and *Plasmodium*, because the TSR domains of SPATRs were clustered in adjacent

230 branches of the phylogenetic tree (Fig. 1a). The old origin of SPATRs may imply their important

231 roles in parasite survival.

232 IFAT and IEM analysis showed TgSPATR was localized in microneme. Additionaly,

233 Ca^{2+}-dependent secretion of TgSPATR was confirmed using ethanol and an intracellular Ca^{2+} chelator.

234 Major microneme proteins, such as MIC2, 4, 10, 11 and AMA1, are secreted in Ca^{2+}-dependent

235 manner, suggesting TgSPATR is a typical microneme protein [12,15,23,24]. Interestingly, the

236 localization of TgSPATR was quite different from PfSPATR, which was localized on the surface of

237 sporozoites and around the rhoptries in asexual erythrocytic stages, but not in any intracellular

238 organelles [20]. TgSPATR is likely to behave and function in a different way from PfSPATRs,

239 because the putative molecular sizes of TgSPATR and PfSPATR are much different (58 kDa and 30

240 kDa, respectively). The specific region of TgSPATR may be a key for targeting micronemes. Two

241 conserved motifs (SYHYY and EIEYE) in the cytoplasmic domain of transmembrane-type MICs are

242 necessary for targeting micronemes, and the propeptide and EGF domains of a soluble protein, MIC3,

243 are important for its localization in micronemes [25,26]. But, TgSPATR does not have such sorting

244 signals, and might be transported into micronemes by a unique recognition mechanism.

245 We succeeded to selectively detect TgSPATR exposed outside parasites, and suggested

246 that TgSPATR was secreted during the early events of invasion. This result suggested a possible

247 function of TgSPATR in the parasite invasion. However, its actual function remains unclear because

248 the anti-TgSPATR antibody used in this research does not inhibit parasite invasion (data not shown).

249 The lack of inhibitory effect may be due to the redundancy of a similar protein, for example MIC2.

250 The amount of MIC2 in A23187-induced secretion is much higher than that of TgSPATR (about
251 11-fold), according to our previous research [19]. Or, TgSPATR may be an escorter that guides other
252 proteins to micronemes and/or parasite surface, like MIC6 and M2AP [11,27]. In this case, the
253 antibody does not inhibit parasite invasion, because TgSPATR has no direct function in invasion.
254 Thus, additional experiments, particularly investigation of over-expression and deletion of the gene,
255 are required to clarify the function of TgSPATR.

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

260

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267

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313 thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is

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335

336 **Figure legends**

337

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

345

346 **Fig. 2. Localization of TgSPATR in micronemes.** Extracellular *T. gondii* was stained using rabbit
347 anti-TgSPATR serum and subsequent Alexa488-labeled secondary antibody (green, b), or using
348 mouse anti-MIC2 monoclonal antibody and Alexa594-labeled secondary antibody (Red, c). Signals
349 of TgSPATR and MIC2 were merged in d. The phase-contrast image corresponds to the fluorescence
350 images (a). In the IEF image of intracellular parasites, arrows indicate signals in slightly dense
351 structures of the parasite apical end (e). These structures are probably micronemes, not rhoptries (R).

352

353 **Fig. 3. Calcium-dependent secretion of TgSPATR.** TgSPATR, MIC2, and β-actin, secreted from
354 parasites, were detected by using western blot analysis. Parasites were pretreated with 50 μM
355 BAPTA-AM (+) or not (–), and then treated with 1% ethanol (+) or not (–). MIC2 was a positive control
356 of Ca^{2+}-dependent secretion, while β-actin was a negative control, indicating the degree of parasite
357 destruction.
358
359 **Fig. 4. Spatiotemporal analysis of TgSPATR secretion.** Intracellular and extracellular parasites
360 were fixed and localization of TgSPATR (a) and MIC2 (b) were analyzed by IFAT, using mouse
361 anti-TgSPATR serum and mouse anti-MIC2 monoclonal antibody respectively. The preparations
362 were permeabilized by 0.002% saponin to see the existence of TgSPATR and MIC2 on the outer
363 surface of parasite. Or, they were permeabilized by 0.5% saponin to see the distribution of the
364 proteins in both outside and inside parasite. The phase-contrast and fluorescence images are merged
365 and arrows indicate the intracellular parasites lacking in TgSPATR or MIC2 on outer surface of
366 parasites.