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Short communication

A transmembrane domain containing pellicle protein of *Toxoplasma gondii* enhances virulence and invasion after extracellular stress[☆]

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ABSTRACT

To identify *Toxoplasma gondii* genes important in the establishment of a persistent infection, we previously used signature-tagged mutagenesis to identify mutants with reduced cyst numbers in the brains of mice. One of the mutants, 95C5, has an insertion within a predicted six transmembrane domain protein, which localizes to the parasite pellicle, thus we named it transmembrane pellicle protein 1 (TgTPP1). Although the 95C5 mutant was found be reduced in its ability to form brain cysts, it is defective during acute infection. Addition of TgTPP1 expressed from its endogenous promoter restored the acute lethality of the 95C5 mutant to parental levels. The 95C5 mutant does not have a growth defect in standard tissue culture conditions; however, we found a significant defect in host cell penetration after extracellular stress. Overall, TgTPP1 may function during acute infection by enhancing the parasites ability to invade after extracellular stress.

1. Introduction, results and discussion

Toxoplasma gondii is an obligate intracellular parasite that has the ability to invade any nucleated cell in its host including immune cells. Host cell invasion by *T. gondii* is rapid (<1 min) and relies on a sequential discharge of a set of specialized secretory organelles, the micronemes, the rhoptries, and the dense granules. Invasion is an active process that is facilitated by an actin-dependent myosin motor found within the parasite pellicle, a unique triple bilayer structure composed of the plasma membrane and the inner membrane complex [1]. The myosin motor is also associated with short actin microfilaments that partner with the glycolytic enzyme aldolase to link the complex with the parasite's plasma membrane through interactions with transmembrane microneme protein (MIC) complexes [2]. These transmembrane MIC complexes are believed to provide the forward motion of motility by binding host cell receptors, facilitating the mechanical force generated by the myosin motor [3].

Several *T. gondii* surface proteins have been shown to be vital for the pathogenesis of the parasite. Disruption of surface antigen 3 (SAG3) causes a two-fold reduction in the ability of the parasite to invade host cells [4]. Surface adhesins such as microneme proteins 1, 2, 3 and 8 (MIC1 [5], MIC2 [6], MIC3 [5] and MIC8 [7], respectively), MIC2-associated protein (M2AP [6]), and apical membrane antigen 1 (AMA1 [8]) are also important for productive infection by *T. gondii*. In previous work, we created of a library of signature-tagged mutants (STM) to discover additional genes that may play a role in the establishment of a persistent infection by *T. gondii* [9]. Here we report the characterization of one of the mutants from this screen, called 95C5. The protein disrupted in 95C5 contains multiple transmembrane domains and is localized to the parasite pellicle. Disruption of this transmembrane domain protein in 95C5 attenuates acute virulence in mice and decreases invasion efficiency after extracellular stress.

To characterize the gene disrupted in the 95C5 mutant, we first mapped the insertion site of the mutagenesis plasmid to the fifth predicted intron of the annotated gene TGME49_051410 (www.toxodb.org) on chromosome XII. The open reading frame (ORF) of TGME49_051410 was determined by sequencing cDNA from wild-type (WT) parasites. We determined the full transcript of TGME49_051410 using rapid amplification of cDNA ends (RACE). The 5′ untranslated region (UTR) is 567 nucleotides and contains a 1090 nucleotide intron, while the 3′UTR is 578 nucleotides. In plants, introns in the 5′UTR have been shown to enhance the level of protein expression through a phenomenon called Intron-Mediated Enhancement (IME) [10–12]. IME has not been investigated in *T. gondii* and we have not determined if IME plays any role in the expression of protein from TGME49_051410. The full length mRNA is 7352 nucleotides (Fig. 1A) and produces a predicted protein of 2069 amino acids.

To verify that TGME49_051410 is disrupted in 95C5 parasites, we compared the expression of TGME49_051410 in C5, the parental

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[☆] *Note:* The GenBank accession number for the TgTPP1 nucleotide sequence is BankIt1450213 TgTPP1 JF900403.

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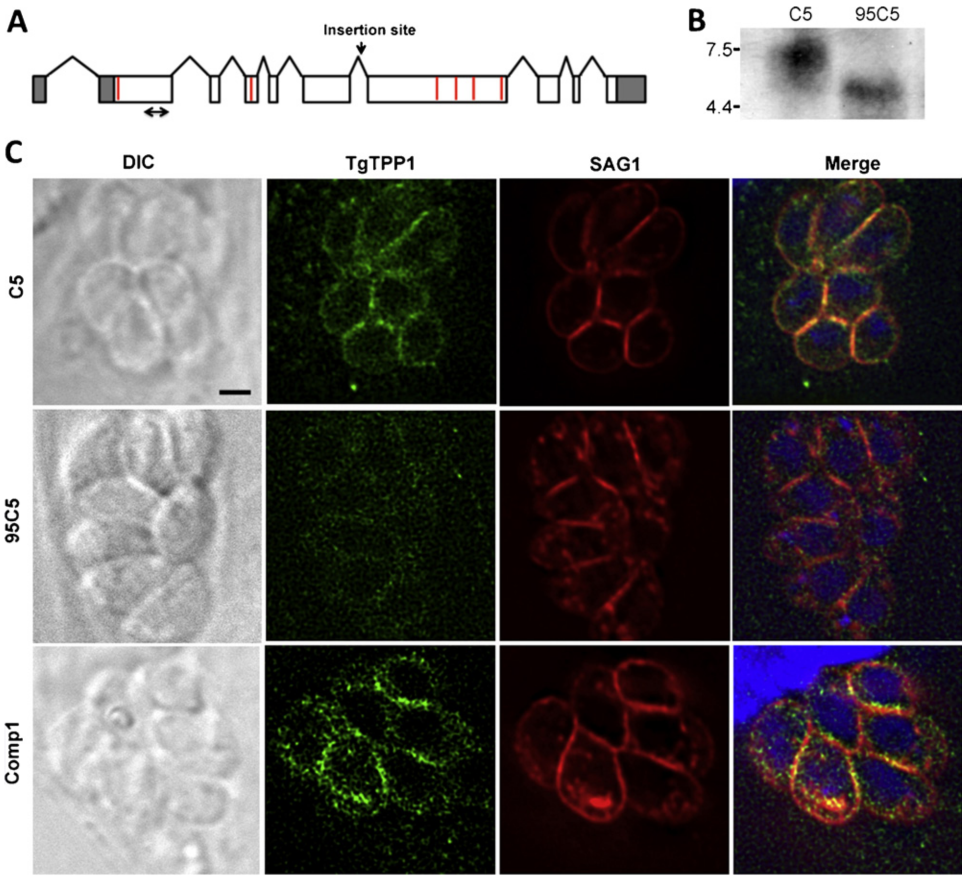

Fig. 1. TgTPP1 is disrupted in the 95C5 mutant and is localized to the parasite surface. (A) A scale drawing of the mapped TGME49_051410 transcript with the UTRs shaded in gray, the exons in white, and the introns indicated by lines above the cartoon. The insertion site in 95C5 is indicated by a downward arrow. The location of the predicted transmembrane domains are indicated by red bars. The probe used in the northern hybridization in B is marked by a double arrow. (B) RNA from C5 and 95C5 parasites was examined by northern hybridization. The numbers to the left indicate the size of the markers of an RNA ladder in kilobases. (C) C5, 95C5 and complemented parasites (Comp1) parasites were seeded onto glass coverslips of human foreskin fibroblasts (HFFs) and allowed to grow for 48 h. The cellular localization of TgTPP1 (green) was visualized using an scFv monoclonal antibody made against a TgTPP1 peptide using the Tomlinson I + J human single fold scFv libraries according to the manufacturer's protocol. The cells were costained for SAG1 (red) and nucleic acid was visualized using 4′6′-diamidino-2-phenylindole (DAPI, blue). The black scale bar equals 2 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

signature-tag strain used to produce 95C5, and 95C5 parasites by northern hybridization. The probe used to detect TGME49_051410 transcript corresponds to the second half of the first exon of TGME49_051410 (double arrow in Fig. 1A). C5 parasites showed a band at the predicted size of 7.3 kilobases while 95C5 parasites showed a significantly smaller message approximately 4.5 kilobases (Fig. 1B). Despite the presence of TGME49_051410 transcript in 95C5, the protein that would be produced from this transcript would be truncated, producing only the first 891 amino acids, or 43% of the predicted protein size.

The TGME49_051410 predicted protein was scanned at the Swiss Institute of Bioinformatics using the BLAST network service (http://www.expasy.org/cgi-bin/blast.pl) but no significant homology was found with any proteins in the database. *Neospora caninum* was the only other Apicomplexan found to have an ortholog. The predicted protein was also analyzed using a variety of other bioinformatics programs to identify potential functional domains. The presence of a signal peptide was predicted using SignalP-HMM ([13,14] http://www.cbs.dtu.dk/services/SignalP/) with a predicted cleavage site between residues 24 and 25. The protein is also predicted to have six transmembrane domains according to TMpred (Fig. 1A, red lines).

To determine the cellular localization of the TGME49_051410 predicted protein, we created an antibody against amino acids 218–329 of the predicted protein using a library of over 100 million different single-chain variable fragments (scFv) from the Tomlinson I and J human single fold scFv libraries (Source BioScience, Nottingham, UK [15]). The cellular localization of the predicted protein was examined by immunofluorescence assay (IFA). In C5 parasites, the predicted protein appears to be in the pellicle of the parasite and colocalized with the surface antigen SAG1 (Fig. 1C, top row). In 95C5 parasites, the staining with the scFv antibody was dramatically less intense compared to C5 parasites (Fig. 1C, middle row). Because of the high level of background staining with the scFv antibody, we verified the localization of the TGME49_051410 predicted protein by genetic complementation of 95C5 with an exogenous copy of TGME49_051410. The expression construct used for complementation includes 2 kilobases upstream of the transcription start site in order to place expression of TGME49_051410 under the control of its own promoter. The construct also includes the intron found in the 5′UTR as well as the first two introns in the coding sequence. The remaining coding sequence was amplified from cDNA due to the large size of the genomic sequence of TGME49_051410 (Table 1). Similar to C5 parasites, complemented 95C5 (Comp1) showed the predicted protein in the pellicle of the parasite, colocalizing with SAG1 (Fig. 1C, bottom row). The peptide used to create the scFv antibody corresponds to a part of the 95C5 protein that is upstream of the insertion site in 95C5 and thus it would detect a shortened form of the protein if it was translated in 95C5 parasites. We attempted to confirm this protein reduction in 95C5 by western immunoblot, however, no signal was detected in either C5 or 95C5 parasites (data not shown). Due to the localiza-