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Isolation of *Toxoplasma gondii* development mutants identifies a potential proteophosphoglycan that enhances cyst wall formation

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Abstract

Within warm-blooded animals, *Toxoplasma gondii* switches from an actively replicating form called a tachyzoite into a slow growing encysted form called a bradyzoite. To uncover the genes involved in bradyzoite development, we screened over 8000 *T. gondii* insertional mutants by immunofluorescence microscopy. We identified nine bradyzoite development mutants that were defective in both cyst wall formation and expression of a bradyzoite specific heat shock protein. One of these mutants, named 42F5, contained an insertion into the predicted gene TGME49_097520. The disrupted protein is serine/proline-rich with homology to proteophosphoglycans from *Leishmania*. *T. gondii* proteophosphoglycan (TgPPG1) expressed from the native promoter was undetectable in tachyzoites, but bradyzoites show punctate spots within the parasite and staining around the parasitophorous vacuole. Complementation of the 42F5 mutant with TgPPG1 expressed from either the α -tubulin or native promoter restores cyst wall formation. Overall, TgPPG1 is upregulated in bradyzoites and enhances cyst wall component expression and assembly.

Keywords

Toxoplasma gondii; development; bradyzoite; cyst wall; proteophosphoglycan

1. Introduction, Results, and Discussion

Toxoplasma gondii is an obligate intracellular parasite whose sexual cycle is restricted to the feline intestine, while the asexual cycle can occur in all warm-blooded animals. During the asexual cycle, the parasite actively replicates as tachyzoites within a parasitophorous vacuole (PV) until undergoing development into a slow growing, encysted bradyzoite form [1]. In immunocompetent hosts, tachyzoite growth is controlled, but quiescent bradyzoites remain. In immunocompromised patients, tachyzoite replication is unchecked, causing a variety of disease states including ocular toxoplasmosis and encephalitis [2,3]. There is a great interest in understanding tachyzoite to bradyzoite development because bradyzoites are a major source for human transmission [4], and there currently are no treatment options for chronic infection.

To aid in the investigation of bradyzoites, conditions have been discovered that promote tachyzoite to bradyzoite development in tissue culture [reviewed in 5]. Studies of both the

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components of the cyst wall and its assembly have been limited, although it is generally thought that cyst wall formation is an early event in bradyzoite development [5–8]. Electron microscopy studies show the cyst wall matrix components appear to assemble along the inner membrane of the PV [9]. Several dense granule proteins have been shown to associate with the cyst wall matrix, and it is known to be composed of proteins containing carbohydrates such as N-acetylgalactosamine and N-acetylglucosamine. These residues allow visualization of the cyst wall with the lectins *Dolichos biflorus* agglutinin (DBA) and succinylated wheat-germ agglutinin (WGA), respectively [10,11]. DBA has been characterized as binding the cyst wall protein, called CST1, a 116kDa bradyzoite specific glycoprotein. A 48kDa protein was identified that binds WGA [9].

This current study screened a library of 8,580 insertional mutants [12] in a tissue culture bradyzoite development assay using FITC-conjugated DBA as a marker for the cyst wall. Mutants were selected for further analysis if they showed reduced cyst wall reactivity after three rounds of qualitative screening. Those selected were analyzed for unique insertion patterns of the mutagenic vector by Southern hybridization. Nine independent mutants were identified as defective in cyst wall formation. Bradyzoite development of these nine mutants was then quantified by immunofluorescence microscopy, using both DBA and the bradyzoite specific heat shock protein BAG1 as hallmarks of development. After three days in bradyzoite conditions, wild type parasites averaged 88% complete cyst wall formation, and 77% BAG1 positive vacuoles, whereas the nine mutants ranged from 8 to 68% complete cyst wall and 7 to 53% BAG1 positive vacuoles (Fig. 1). Examples of staining for wild type and mutants 42F5, 76E2, and N28E2 are shown (Fig. S1). At three days post-bradyzoite induction, N28E2 consistently had only 1–2 parasites per vacuole. These vacuoles contained space without parasites, rather than having a vacuolar membrane tightly surrounding the parasites.

None of the development mutants (Fig. 1) were completely unable to form bradyzoites. This result was not surprising since a library of 8,580 insertional mutants does not represent saturation of a nearly 60 megabase genome. Also, there are likely redundant pathways for bradyzoite development present in *T. gondii*. Microarray analysis has shown the complexity of bradyzoite development [12 and www.toxoDB.org]. Other bradyzoite development screens with mutant libraries having greater genomic coverage due to chemical mutagenesis strategies have noted the inability to obtain mutants completely disrupted in bradyzoite development. Researchers have hypothesized that these type of mutants may be non-viable as tachyzoites, due to shared genetic pathways, or that there are ways to circumvent any disruption in the bradyzoite development pathway, albeit at a lower frequency [6,12–14].

Although not necessary for this bradyzoite development screen, each mutant from the library contains two independently inserted plasmids. The first insertion was a signature tag-bearing plasmid. Sixty unique tagged strains then served as parent strains for further mutagenesis by insertion of plasmid pLK47 [15]. The insertion site of the pLK47 was determined for four of the nine mutants (Table 1). In some cases, the frequency of mutants derived from the same parental strain led to suspicions that the original insertion within the parent strains was responsible for the *in vitro* bradyzoite defect. It was found that the parental strains C9 and E6 for mutants 44C9, 42C9, 47E6, and 72E6 were defective in tissue culture bradyzoite development (data not shown). Therefore, the genes disrupted in the parental strains, C9 and E6, are listed in Table 1. The insertion sites of 47E6 and N28E2 have not been identified because either the pLK47 plasmid fragmented upon insertion, or an electroporation-induced mutation was generated. The insertion site of 76E2 disrupts the *GRA3* locus, but it was determined that disruption of *GRA3* does not effect bradyzoite development [16]. Mutant 42F5 was selected from the nine mutants for further study because it has a strong defect in bradyzoite development, the genomic site disrupted by the insertional plasmid was identified, and the F5 parental strain is not defective in tissue culture bradyzoite formation (Fig. 2 and S2).

The pLK47 plasmid in mutant 42F5 inserted within the coding region of predicted gene TGME49_097520 (Fig. S3A, lollipop). Exons and introns were identified for TGME49_097520 by amplification of the entire coding region using primers based on the gene prediction (www.toxoDB.org). The identified genomic locus spans approximately 11.5 kb comprised of 15 exons encoding an open reading frame (ORF) of 4,569 bp (Fig. S3A). TGME49_097520 encodes a hypothetical protein of 1,522 amino acids and 163 kDa. One defining characteristic of this protein is an abundance of hydrophilic amino acid residues, as illustrated by the Kyte-Doolittle hydrophobicity plot (Fig. S3B). The insertion site in 42F5 is within exon 12 of the genomic locus. Rapid amplification of cDNA ends (RACE) was used to identify the 5' and 3' ends of the mature transcript. The 5' untranslated region (UTR) is 944 bp in length, and contains a consensus transcriptional start site for *T. gondii* [17]. The 3' RACE reaction identified two polyadenylation sites, resulting in 3'UTRs of 202 bp and 383 bp in length. The total mRNA transcript size is therefore either 5,715 bp or 5,896 bp.

Disruption of TGME49_097520 was confirmed by northern hybridization. Probes upstream and downstream of the insertion site (diagramed in Fig. S3A) were used to examine the mRNA in the 42F5 mutant in compared to wild type. A probe to the 3' end of the gene shows complete loss of transcript in 42F5 after the mutational insertion site; however, a probe to the 5' end of the gene identified a transcript in 42F5 that is larger than that seen in the wild type (Fig. S3C). This larger transcript is likely due to read through into the pLK47 insertional plasmid, suggesting a truncated form of the protein may be translated in 42F5.

The proteophosphoglycans (PPG) from *Leishmania* aid parasite survival in different host environments throughout its developmental life cycle [18,19]. BLAST searches noted homology of TGME49_097520 to PPGs from *Leishmania* spp. Further analysis showed that the N- and C-terminal thirds of the protein have a high percentage of serine (24 and 19%, respectively). The central third of the protein is highly enriched in charged amino acids, including over 20% acidic residues. Interestingly, proline is 10% of the amino acids in N- and C-terminal serine-rich domains, but less than 1% of the central charged domain. The N- and C-terminal thirds of the protein contain several serine/proline-rich repeat sequences that are similar to the serine/proline-rich repeats of *Leishmania* [19]. Four examples of these domains from the N-terminus are shown (Fig. S3D). We named this protein *T. gondii* proteophosphoglycan (TgPPG1). Six other *T. gondii* proteins with 25–38% identity to *Leishmania* PPGs have been identified by proteomic analysis of a type I strain parasitophorous vacuole membrane (PVM). The smallest of these PPGs was epitope tagged and localized to the dense granules and PVM of tachyzoites (A. Sinai, personal communications).

Leishmania PPGs are highly glycosylated and cannot be separated by SDS-polyacrylamide gel electrophoresis [18]. TgPPG1 is annotated as having a signal peptide (www.toxodb.org and SignalP-HMM <http://www.cbs.dtu.dk/services/SignalP/>), allowing access to glycosylation machinery. In *T. gondii* expressing TgPPG1 from its native promoter with a C-terminal HA tag, no distinct bands could be seen by SDS-PAGE in either tachyzoite or bradyzoite conditions. However, bradyzoites consistently had a low level of reactivity that did not enter the resolving gel (data not shown), indicating that TgPPG1 maybe highly glycosylated.

To confirm that disruption of TgPPG1 was responsible for the observed bradyzoite switch defect, the 42F5 mutant was genetically engineered to express the wild type ORF, with or without a C-terminal hemagglutinin tag (HA). Clones expressing TgPPG1 from the endogenous (ENDO, ENDOHA1, ENDOHA2) or α -tubulin promoter (TUBHA) restored *in vitro* cyst wall formation to near wild type levels at both two and three days in bradyzoite inducing conditions (Fig. 2A). The variation in levels of complementation is not surprising due to the random integration of each construct by non-homologous recombination into the *T. gondii* genome. BAG1 expression was not complemented by exogenous expression of TgPPG1

(Fig. S2). These results point to a role for TgPPG1 in cyst wall formation. The lack of restoration of BAG1 expression indicates that 42F5 may possess another defect not caused by pLK47. The BAG1 expression in the parental strain F5 is similar to wild type (Fig. S2).

The phenotype of 42F5 is more pronounced earlier in the bradyzoite development process (two days, Fig. 2A). When the 42F5 mutant is placed under harsh conditions, such as being extracellular for several hours, the phenotype is not as pronounced after a three day *in vitro* switch (data not shown). It appears that disruption of *TgPPG1* in the 42F5 mutant delays cyst wall assembly, but the mutant is capable of forming complete cyst walls to near wild type levels eventually. It is unknown if truncated protein is translated from the partial transcript present in 42F5. The ability of the mutant to eventually form complete cyst walls may indicate that truncated TgPPG1 is translated in 42F5 and partially functional. Multiple attempts to delete TgPPG1 in a wild type background were unsuccessful, suggesting that a small amount of TgPPG1 is synthesized in tachyzoites and that the lack of the entire protein is lethal.

DBA has been characterized to bind N-acetylgalactosamine residues on the bradyzoite cyst wall marker, CST1 [9]. Along with a reduction in complete cyst walls as judged by DBA, 42F5 bradyzoites had lower CST1 staining by immunofluorescence than parental F5 bradyzoites (data not shown). In order to quantitatively assess differences in CST1 protein levels between the parental F5, 42F5, ENDO, and TUBHA bradyzoites, a western immunoblot was performed (Fig. 2B). The mutant 42F5 was decreased in CST1 levels in bradyzoites compared to F5 validating the observed decrease in complete cyst wall formation by visual quantification. The two complemented strains, ENDO and TUBHA, were restored in CST1 levels by western immunoblot (Fig. 2B).

To assist in understanding the function of TgPPG1, its localization was examined. TgPPG1 expressed from its native promoter (ENDOHA1) was not apparent in tachyzoites by immunofluorescence (data not shown). However, TgPPG1 expressed in ENDOHA1 bradyzoites is evident in faint punctate spots and appears around the PVM where it co-localizes with the cyst wall marker DBA (Fig. 2C). The bradyzoite localization of TgPPG1 suggests its association with the developing cyst wall.

How TgPPG1 affects cyst wall assembly and CST1 expression is currently unknown. Secretion of cyst wall has been described as an early differentiation event with components visualized as soon as 24 hours after exposure to bradyzoite inducing conditions [6–9]. TgPPG1 may be an escorter protein and its disruption prevents the release of cyst wall components. Alternatively, TgPPG1 could mediate signaling. The vast exocytosis occurring early in bradyzoite development needs to be compensated by equal endocytosis. In other developmental models, endocytic trafficking leads to Notch signaling that directs transcriptional regulation of nuclear genes [reviewed in 20]. These two models are not necessarily exclusive, as TgPPG1 escort of cyst wall components could lead to nuclear signaling through the Notch pathway.

In summary, this study used a visual screen to identify nine mutants that were defective in cyst wall formation. Further characterization of one of the proteins with homology to PPGs shows that that protein localizes to the PVM in bradyzoite conditions and enhances cyst wall formation. As the PVM likely serves as the foundation for the bradyzoite cyst wall, the movement of TgPPG1 to the PVM in bradyzoites aligns with its functional role of enhancing cyst wall development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Bradyzoite development screen identified nine mutants. Wild type (PRU) and potential bradyzoite development mutants (x-axis) were grown in bradyzoite inducing conditions for three days [14]. Cells were fixed and stained with DBA to mark the cyst wall (black bars) and the bradyzoite marker BAG1 (white bars). The percentage of complete cyst wall formation and BAG1 positive vacuoles are shown on the y-axis. Three sets of fifty vacuoles were counted for each of three independent experiments and reported as the average with standard error indicated.

Fig. 2.

Complementation of the 42F5 mutant restores *in vitro* cyst wall formation. (A) TgPPG1 expressed from either its native (ENDO, ENDOHA1, ENDOHA2) or constitutive α -tubulin (TUBHA) promoter, with or without a C-terminal HA epitope tag, restored complete cyst wall formation to 42F5 after two or three days in bradyzoite inducing conditions. Results are reported as the average of two independent experiments with standard deviation. (B) Western immunoblot of F5, 42F5, ENDO, and TUBHA bradyzoite protein lysates probed with mouse anti-CST1 and rabbit anti- β -tubulin as a loading control. (C) *T. gondii* expressing TgPPG1 from its native promoter (ENDOHA1) were grown in bradyzoite inducing conditions for 5 days. Cells were fixed and stained with an anti-HA antibody to visualize TgPPG1 (green) and

DBA to mark the cyst wall (red). All coverslips were mounted with VectaShield mounting medium containing 4'6'-diamidino-2-phenylindole (DAPI, Vector Laboratories) to visualize parasite nuclei (blue), seen in the merged image. Fluorescent images were acquired as previously described [15]. The size of the white scale bar in the merged image equals 5 μm .

Table 1

Insertion sites for Bradyzoite Development Mutants. Insertion sites and disrupted genes were identified using methods previously described [20].

Mutant	Chromosome: Insertion site (bp)	Predicted annotations
42F5	II: 1,849,217	TGME49_097520: hypothetical protein
49F5	VI: 2,579,711	TGME49_043400: PX domain-containing protein
72F2	IV: 309,198	TGME49_120450: ribosome biogenesis regulatory protein homologue, RRS1
76E2	X: 992,067	TGME49_027280: GRA3
N28E2	Unknown	N/A
C9	V: 1,576,893	TGME49_086920: chromatin structure remodeling complex protein, RSC8
E6	VIIb: 1,785,558	TGME49_061370: hypothetical protein TGME49_061260: jmjC domain-containing protein