Published in final edited form as:

Mol Microbiol. 2016 December ; 102(6): 1086–1098. doi:10.1111/mmi.13538.

Developmental change in translation initiation alters the localization of a common microbial protein necessary for *Toxoplasma* chronic infection

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Summary

The *Toxoplasma gondii* cyst stage is resistant to drug therapy. To identify potential targets for new therapeutics, we screened insertional mutants of *T. gondii* for a reduced ability to form cysts in the brains of mice. In one of these mutants, named 38C3, the mutagenesis plasmid inserted into the mRNA of a protein that is highly conserved in microbes but is not present in humans. The mutation in 38C3 causes reduced brain cyst production during chronic infection, but does not affect acute virulence, so the disrupted gene and protein are called *T. gondii* Brain Colonization Protein 1 (TgBCP1). TgBCP1 has three potential in frame start codons that produce either 51, 33 or 25 kDa proteins. In rapidly replicating tachyzoites, translation initiates at the third methionine, producing the 25 kDa form that is conserved in many bacteria and protozoans. Brain cysts exclusively express the 51 kDa form of TgBCP1, which is secreted from the parasites and localizes to the cyst wall. Only expression of the long form of TgBCP1 restored cyst formation in the 38C3 mutant. TgBCP1 is essential for cyst formation and is the first example of a developmental regulation in translation initiation site preference for a *T. gondii* protein.

Graphical abstract

Abbreviated Summary

A *Toxoplasma* mutant that is disrupted in a protein highly conserved in microbes but is not present in humans, produces fewer cysts in mouse brains during chronic infection. In cell culture, translation of this protein initiates at the third methionine to produce a 25 kDa form, whereas in brain cysts translation begins at the first methionine to produce a 51 kDa form that is secreted from the parasites and localizes to the cyst wall.

Keywords

parasite; *Apicomplexa*; *Toxoplasma*; cyst development; translation initiation

Introduction

The Apicomplexa phylum contains over 5000 species of obligate intracellular parasites, several of which are important pathogens. For example, *Cryptosporidium* parasites are the leading cause of diarrheal disease, which can be fatal in children and immunocompromised adults. Drug development to combat *Cryptosporidium* has been hindered due to the difficulty of propagating and storing *Cryptosporidium* long term (Ryan & Hijjawi 2015). Another Apicomplexan, *Plasmodium* spp., infects more than 150,000,000 people annually and killed over 500,000 people in 2013 (www.cdc.gov/malaria). Development of new drug therapies for *Plasmodium* infections is hindered by the complex life cycle and difficulty in genetically manipulating the parasite. *Toxoplasma gondii* is an Apicomplexan that can invade any nucleated cell within any warm-blooded host, making it one of the most common parasitic infections in the world. This ubiquitous parasite can cause fatal infections in neonates and immunocompromised persons. *T. gondii* is relatively easy to propagate in tissue culture, can be stored long term, and is amenable to genetic manipulation, so it is often considered the model Apicomplexan.

T. gondii parasites maintain two life forms in mammalian hosts. The parasite infects the host in cyst forms from undercooked meat, or as oocysts shed in the feces of a cat. Upon ingestion, *T. gondii* breaks out of the meat cyst or oocyst, and disseminates throughout the body during acute infection as a rapidly replicating form called a tachyzoite. In response to a potent cell mediated immune response, *T. gondii* differentiates into a stable cyst stage called a bradyzoite, the hallmark of chronic infection. Bradyzoite cysts are drug resistant and protected from the immune system because they remain dormant within the cells of the central nervous system and skeletal muscles. The chronic cyst stage can revert to the fast replicating tachyzoite stage when cellular immune surveillance is lost, resulting in toxoplasmic encephalitis in immunocompromised patients. For women who become infected for the first time during pregnancy, *T. gondii* can cross the placental barrier and infect the fetus before an effective adaptive immune response against the parasite can develop. No therapy exists to kill the chronic cyst form, and there is no vaccine safe for use in humans.

To identify potential targets for drugs effective against the bradyzoite stage, we previously performed signature tagged mutagenesis to identify genes necessary for *T. gondii* to form a chronic infection in mice. In this manuscript, we identify the gene disrupted in one of these mutants, named 38C3, and map the mature RNA transcript. We show that expression of this gene is essential for brain cyst formation and we characterize two forms of the protein, called *T. gondii* Brain Colonization Protein 1 (TgBCP1). We compare homologues of TgBCP1 in several other microorganisms, including a number of Apicomplexan parasites. Finally, we describe expression and localization of both forms of TgBCP1 protein during tachyzoite and bradyzoite life stages. While the short form of TgBCP1 is maintained in the cytosol of tachyzoites, the long form is only expressed in parasites during chronic animal infection and localizes to the bradyzoite cyst wall.

Results

38C3 has reduced cyst burden but no defect during acute infection

The *T. gondii* mutant 38C3 was identified as severely defective in establishing chronic infection in mice during a modified signature tagged mutagenesis (STM) screen (Frankel *et al.* 2007). We previously showed that the signature tagged parental strain of the mutant 38C3, called C3, has an insertion in the promoter of the ribosome biogenesis regulatory protein (TgRRS1, Fig. S1) and a defect in the ability to form cysts in the brain (Milligan-Myhre *et al.* 2011). However, of the 105 mutants in the library containing the C3 parental tag, only three were defective enough to be uncovered in the STM screen (Frankel *et al.* 2007). Therefore we hypothesized that the 38C3 mutant has an additive defect in brain cyst formation and/or maintenance. To test this hypothesis, we inoculated mice with the 38C3 strain, the parental C3 strain, or the wild-type Pru strain lacking the hypoxanthine-xanthine-guanine phosphoribosyl transferase (Pru⁻) that C3 was derived from. While infection with the C3 strain produced fewer cysts than Pru⁻ ($p < 0.05$), the 38C3 mutant had a greater defect in cyst formation than the C3 parental strain ($p < 0.01$, Fig. 1A). We then compared the lethality of the 38C3 and C3 parasites to Pru⁻ during acute infection. Pru⁻ was uniformly lethal after infection with 4×10^5 (Fig. 1B) or 10^6 (Fig. 1C) parasites. C3 and 38C3 parasites had reduced virulence at both doses compared to Pru⁻, but were not significantly different

from each other in acute infection virulence at either high dose. These results indicate that the first insertion in the C3 parental strain affects acute virulence of the parasites, while the second insertion site in 38C3 mutant affects the parasites ability to form and/or maintain chronic infection cysts.

38C3 has an insertion in the gene annotated TGME49_203450

We identified the DNA flanking the insertion site for the 38C3 mutant by plasmid rescue, as described previously (Frankel *et al.* 2007; Craver *et al.* 2009; Milligan-Myhre *et al.* 2011). 38C3 has a plasmid insertion in the Not I site within the predicted open reading frame (ORF) of gene TGME49_203450, located on chromosome VIIa of the sequenced *T. gondii* ME49 genome from 2,493,933 to 2,500,272 (Fig. 2A and S1). TGME49_203450 is expressed in all phases of the tachyzoite cell cycle and in both intracellular and extracellular parasites (<http://toxodb.org/toxo/>). To confirm that mRNA is expressed in tachyzoites and disrupted in the 38C3 mutant, we probed a membrane containing tachyzoite RNA from Pru, the 38C3 mutant, and the parent strain C3. The 2.3 kb mRNA was present in Pru and the C3 parental strain, but absent from the 38C3 mutant, confirming that the mutagenesis plasmid inserted into the TGME49_203450 mRNA in the 38C3 mutant (Fig. 2B). Given that the disruption of TGME49_203450 in the 38C3 mutant causes a defect in brain cyst burden, but not acute virulence, we called the gene and the resulting protein *T. gondii* Brain Colonization Protein 1 (TgBCP1).

Because we had previously observed that transcription continues through the insertion plasmid in some of our STM mutants (Rooney *et al.* 2011a and b), we examined the TgBCP1 mRNA in the 38C3 mutant by denaturing Northern blot, which facilitates the transfer of large mRNAs. While the C3 strain contained the 2.3 kb *TgBCP1* transcript, NaOH treatment of the gel allowed a greater than 9 kb mRNA containing the TgBCP1 to transfer from the lane containing 38C3 RNA (Fig. 2C). These results show that the TgBCP1 locus is disrupted in the 38C3 mutant; however, part of the ORF may still be intact and available for translation.

Identification of the translational start site

The TgBCP1 ORF contains three potential initiator methionines (Fig. 2A). The third in frame ATG has the best *T. gondii* Kozak-like consensus translation initiation sequence (Seeber 1997). An alignment of TgBCP1 protein with the orthologues from other Apicomplexans showed significant homology only when translation initiates at the third ATG (Fig. 3). Analysis of the MS/MS peptide data (<http://toxodb.org/toxo>) shows that while many peptides map to TgBCP1, no tachyzoite peptides map upstream of the third ATG in the ORF. Thus, we predicted translation would begin at the third in frame methionine. To determine which start codon of TgBCP1 is used, we designed two constructs expressing TgBCP1 with a C-terminal hemagglutinin (HA) tag. One construct, called Endo:TgBCP1-HA, contained the entire endogenous 5'UTR and approximately 1700 bp upstream of the *TgBCP1* transcription start site, which would likely include the *TgBCP1* promoter (Fig. 4A). This construct would allow the ribosomal subunits to initiate translation with the start codon preferred by the translation system. The second construct, called Best Kozak:TgBCP1-HA, contained the ORF starting at the third in frame ATG expressed from

the constitutively-active α -tubulin promoter and 5'UTR (Fig. 4B). We analyzed western blots of tachyzoite lysates of Best Kozak:TgBCP1-HA and Endo:TgBCP1-HA clones probed with anti-HA antibodies. We saw that translation of TgBCP1 in tachyzoites likely begins at the third ATG in the ORF as the western blot reveals that both the Best Kozak:TgBCP1-HA and Endo:TgBCP1-HA strains expressed an approximately 25 kDa HA-tagged protein (Fig. 4C). Both strains also expressed a slightly lower band, more prominent in the Best Kozak:TgBCP1-HA due to over-expression, which may be a processed form of the protein.

To examine the molecular weight of TgBCP1 in C3 and 38C3 strains, we generated polyclonal antibodies to the 25 kDa form of TgBCP1. Western blot analysis showed that a 25 kDa TgBCP1 was present in all strain, including the 38C3 mutant (Fig. 4D), corroborating the previous data that translation begins at the third in frame methionine. This result also shows that translation of TgBCP1 at or near parental levels occurs from the STM plasmid fusion mRNA present in the 38C3 mutant. The specificity of the TgBCP1 antibody was demonstrated by the presence of the a slightly larger band in the Best Kozak:TgBCP1-HA and Endo:TgBCP1-HA expressing strains. Probing these blots with anti-HA antibody showed a single approximately 26 kDa band in the Best Kozak:TgBCP1-HA and Endo:TgBCP1-HA expressing strains (Fig. 4D). These data show that translation occurs at the third in frame methionine and the 25 kDa form of TgBCP1 is still present in the 38C3 mutant. While the majority of parasites grown in tissue culture bradyzoite conditions expressed only the 25 kDa form of TgBCP1, an occasional bradyzoite experiment showed a 50 kDa form of TgBCP1 (Fig. S2A and B). The presence of the 50 kDa form of TgBCP1 in a few tissue culture bradyzoite development experiments highlights that TgBCP1 may change the site of translation initiation during chronic infection.

Localization of the TgBCP1 in tissue culture tachyzoites and bradyzoites

The 25 kDa TgBCP1 protein is not predicted to contain a signal sequence and is likely distributed throughout the parasite. Using the anti-TgBCP1 antibody, we saw the TgBCP1 dispersed throughout the parasite cytoplasm, often with punctate staining that was distinct from characterized parasite organelles including the dense granules as marked by dense granule protein 2 (GRA2) and the plant like vacuole as marked by Na(+)/H(+) exchanger (NHE3). Localization of TgBCP1 was similar between Pru (Fig. S3), C3 and 38C3 (Fig. 5) strains, and in parasites grown in tachyzoite and bradyzoite conditions. In tissue culture bradyzoites, TgBCP1 appears to partially co-localize with cytoplasmic bradyzoite-specific heat shock protein BAG1 (Bohne et al., 1995, Parmley et al., 1995) but not co-localize with the cyst wall lectin Dolichos biflorus agglutinin (DBA). We also analyzed the location of Endo:TgBCP1-HA and Best Kozak:TgBCP1-HA grown in tissue culture tachyzoite and bradyzoite conditions, then stained with either anti-HA or anti-TgBCP1 antibodies. We found similar localization of TgBCP1 in these strains with either antibody (Fig. S4). Along with highlighting that the localization TgBCP1 was not affected in the 38C3 mutant, these results show that the 38C3 was able to differentiate to bradyzoite cysts under tissue culture conditions.

Complementation of mutant 38C3 with TgBCP1 restores cyst formation in the brains of mice

Given that the 38C3 mutant has reduced cysts in the brains of chronically infected mice and *TgBCP1* was disrupted in the mutant, we complemented the 38C3 mutant with either Endo:TgBCP1-HA or Best Kozak:TgBCP1-HA and examined the number of cysts per infected brain. Multiple transfections were performed for each construct to ensure that each clone contained a unique insertion event that did not influence expression. We found that the Endo:TgBCP1-HA expressing clones were able to restore the ability of 38C3 to form cysts in the brains of mice to the level comparable to the parental strain C3 (Fig. 6A, $p < 0.0001$ for C3 and 38C3 as well as 38C3 and Endo1, $p < 0.005$ for 38C3 and Endo2). While complementation with Endo:TgBCP1-HA restored cyst counts to near parental levels, expression of TgBCP1 from the Best Kozak:TgBCP1-HA did not increase cyst counts (Fig. 6). Two possible reasons for this complete lack of complementation were 1) overexpression of Best Kozak:TgBCP1-HA from the α -tubulin promoter was detrimental to cyst formation or 2) in brains cysts, translation of endogenous TgBCP1 begins at the first in frame ATG, which is not possible from the Best Kozak:TgBCP1-HA construct.

Change in translation initiation site of TgBCP1 in brain cysts

To test the hypothesis that TgBCP1 was translated from the first in frame ATG during chronic infection, we compared the size of the TgBCP1 expressed in bradyzoites isolated from brain cysts. Due to the low number of cysts formed by both 38C3 or the parent strain C3, we examined protein expression in the Pru strain. We directly compared the size of the TgBCP1 expressed in Pru tissue culture tachyzoites and cysts percoll purified from the brain 28 days post-infection. We saw a 25 kDa band in tissue culture derived tachyzoites, which was no longer present in brain cysts purified from either CBA/J mice (Fig. 6B) or C57BL/6 mice (Fig. S2C). Instead, brain cysts express an approximately 50 kDa form of TgBCP1, which is indicative of translation initiation switching to the first in frame methionine. We also saw that the TgBCP1 mRNA from purified brain cysts was not spliced alternatively compared to the tissue culture tachyzoite stage. These results suggest that the preferred site of initiation for TgBCP1 translation changes in during chronic animal infection. Because the Best Kozak:TgBCP1-HA did not complement the 38C3 mutant cyst formation defect, it is likely that this 50 kDa form of TgBCP1 is necessary for cyst formation.

Localization of the TgBCP1 in brain cysts

Translation from the first ORF would result in a 50.7 kDa protein that is predicted by the SignalP program (Petersen *et al.* 2011) to contain an endoplasmic reticulum (ER)-type signal sequence. We examined the localization of TgBCP1 in Pru brain cysts using the anti-TgBCP1 antibody. TgBCP1 appears to be secreted from the parasites, not co-localizing with BAG1 but partially co-localizing with DBA on the cyst wall (Fig. 7). Multiple cysts of different sizes all show the TgBCP1 protein no longer within the individual bradyzoites, but instead within the parasitophorous vacuole space and along the cyst wall. We also examined the localization of TgBCP1 expressed from the Endo:TgBCP1-HA construct in purified mouse brain cysts that were stained with anti-HA antibodies. Similar to the Pru brain cysts

stained with anti-TgBCP1 antibody, we saw TgBCP1 localized to the cyst wall with DBA in Endo:TgBCP1-HA expressing brain cysts stained with anti-HA (Fig. 7). As expected, no specific staining was seen in Pru⁻ purified brain cysts using the anti-HA antibody (Fig. 7). Overall, these results show that TgBCP1 changes localization from within the parasite in tissue culture tachyzoites to outside the parasite and along the cyst wall in mouse brain bradyzoite cysts. These results also show that tissue culture bradyzoite development conditions do not exactly model chronic animal infection.

Discussion

We previously used STM to identify 40 insertional mutants that showed at least a 10-fold decrease in cyst burden compared to Pru⁻ parasites. In this STM screen, we compared blots from pools of parasites simultaneously passaged in tissue culture for 22 days, allowing us to isolate genes essential only during growth in animals. Approximately half of the disrupted genes encode proteins without predicted function and most of these hypothetical proteins are conserved within other organisms. Using *T. gondii* as a model to define the mechanism of virulence for these hypothetical proteins will provide new insight and potential drug targets applicable for several pathogens. 38C3 was one of those insertional mutants disrupted in a hypothetical protein that was conserved within many microbes. In this manuscript, we characterized the gene disrupted in the 38C3 mutant and the two TgBCP1 proteins translated from it.

Initiation of TgBCP1 occurs at the third in frame methionine in tissue culture tachyzoites, but at the first AUG in bradyzoite cysts during chronic infection. While translation did occur at the first AUG in an occasional tissue culture bradyzoite development experiment (Fig. S2), the majority of tissue culture experiments showed only the 25 kDa form of TgBCP1 (Fig. 4D). These results highlight that while the tissue culture model of bradyzoite development is an outstanding model, it does not perfectly recapitulate chronic animal infection. Also shown in figure 4D is that in the 38C3 mutant, TgBCP1 is translated at or near wild type levels from the third AUG of the large fusion mRNA. Translation from this fusion mRNA suggests that the third AUG is not selected by a scanning mechanism, but instead it is likely translated by an Internal Ribosomal Entry Sequence (IRES). It may also be important for the 38C3 phenotype that there is an additional mutation in the ribosome biogenesis regulatory protein (TgRRS1) in the C3 parental strain (Milligan-Myhre *et al.* 2011). TgRRS1 could be affecting the translation of the TgBCP1 and/or the TgRRS1 mutation is necessary for the chronic infection phenotype seen for TgBCP1. Future studies will examine the potential IRES-mediated translation of TgBCP1 and the contribution of TgRRS1 to that mechanism.

The TgBCP1 protein is homologous with other members of the Apicomplexa phylum beginning only after the third in frame methionine possibly because most Apicomplexans do not contain a tissue cyst stage analogous to the bradyzoite stage of *T. gondii* chronic infection. One exception is *Hammondia hammondi*, an Apicomplexan closely related to *T. gondii* that also has a chronic cyst stage and protein homology starting at the first in frame TgBCP1 methionine. In *H. hammondi*, the TgBCP1 protein is predicted to initiate translation before the first methionine of TgBCP1, but translation at this methionine does not

encode a protein with an N-terminal (ER)-type signal sequence (Fig. 3). If translation of the *H. hammondi* protein begins at the second in frame AUG, this protein contains a signal sequence as well as high degree of conservation to TgBCP1 throughout the entire protein. It is interesting that this N-terminal bradyzoite-specific half of TgBCP1 is serine and proline-rich, 47 and 35% respectively, similar to the proteophosphoglycan protein that localizes to the cyst wall to enhance its formation (Craver *et al.*, 2010).

This developmental change in translation initiation alters the localization of TgBCP1 from within tachyzoites to outside bradyzoites in the parasitophorous vacuole space and cyst wall. The *T. gondii* cGMP dependent protein kinase (PKG) also changes size and intracellular localization depending on which potential initiator methionine is used (Donald and Liberator 2002). The 110 kDa form of *T. gondii* PKG associates with the parasite plasma membrane, whereas the 100 kDa form is cytosolic; however, it has not yet been determined whether the initiator methionine preferences are developmentally regulated in the tachyzoite to bradyzoite transition. An example of a protein changing localization in response to bradyzoite development is the patatin-like phospholipase TgPL1. In tachyzoites, TgPL1 is localized to vesicles inside the parasite that are distinct from the dense granules, but in the encysted bradyzoite stage, TgPL1 is localized to the parasitophorous vacuole and cyst wall (Magle *et al.*, 2014). Translation of TgPL1 begins at the same methionine, giving an ER-type signal sequence in both tachyzoites and bradyzoites, so TgPL1 localization change is not dependent on differences in translation initiation. Instead, it appears that the tachyzoite vesicles that TgPL1 is localized to are secreted during stress responses and bradyzoite development (Magle *et al.*, 2014, Tobin *et al.*, 2012). The identity of these tachyzoite vesicles and mechanism of secretion are still unknown, but will be important avenues of future research.

In the type II strain, *TgBCP1* transcript is expressed during both acute and chronic mice infections (Pittman *et al.* 2014), throughout infection within the feline intestinal epithelium (Hehl *et al.* 2015), as well as in unsporulated and sporulated parasites (Fritz, *et al.*, 2012). Future work will determine the size and location of TgBCP1 in the sexual stages and if it localizes to the oocyst wall. While no MS/MS peptides map upstream of the third ATG in TgBCP1 in oocysts, there are only two peptides that map to TgBCP1 in the two oocyst proteomes (<http://toxodb.org/toxo/>), so more oocyst peptide data is needed. Similarly, analysis of all of the MS/MS peptide data available on ToxoDB.org shows no peptides upstream of the third ATG in the ORF; however, no mass spectrometry peptide databases have been generated from the bradyzoite stage. This lack of data highlights the need for a bradyzoite proteomics database, especially one generated from chronic infection cysts. Addition of a bradyzoite peptide database will allow for analysis of the preferred sequence context for translation of bradyzoite proteins. Perhaps the preferred translational start site changes for other proteins during bradyzoite development.

We compared the predicted protein structure to the NCBI protein database and determined that TgBCP1 homologues are present in microbes across several Phyla, including Chlorophyta, Oomycota and Proteobacteria (Fig. S5). Additionally, the *TgBCP1* gene is highly conserved in all six sequenced *T. gondii* lineages (<http://toxodb.org/toxo/>). A search for conserved domains in the TgBCP1 protein revealed that TgBCP1 is a member of the

DUF3228 protein family, although the mechanism of action of this family of proteins has not been described. While TgBCP1 homologues are present in microbes across a wide variety of Phyla, none have been well characterized. The TgBCP1 homologue in the green algae *Chlamydomonas reinhardtii* is described as a flagellar associated protein (FAP25, accession number XM_001692099.1) (Pazour *et al.*, 2005). Although several screens have been performed in *C. reinhardtii* to identify genes and proteins involved with flagella, FAP25 was identified in only one (Merchant *et al.*, 2007, Finst *et al.*, 1998, Smith and Lefebvre 1997). In addition, FAP25 flagellar localization was not confirmed by a second method, and at least two cytoplasmic proteins were identified as being associated with the flagellar matrix in the study. Therefore, it cannot be definitively concluded that FAP25 is a flagellar protein, and further characterization of FAP25 has not been performed.

Both TgBCP1 and the ortholog from *C. parvum* have been crystalized as part of the efforts to determine the structures of proteins from emerging or re-emerging pathogens. Their structures have been deposited in the Center for Structural Genomics of Infectious Diseases (CSGID, both structures at <http://www.csgid.org/csgid/targets/view/IDP90278>). All crystal structures are open to the public, including structure details and methods as well as references to biological function if known. Most of the conserved residues in the TgBCP1 family appear to line a pocket. The CSGID hypothesize that three residues (C53, H55, and D115) form a catalytic triad, and that K154 may interact with an unusually large potential active site with a potential flexible loop nearby. However, an alignment of all hypothetical proteins related to TgBCP1 reveals that, while the positively charged K154 is moderately conserved (26 of 36 proteins have the residue), ten proteins have non-charged or negatively charged residues at that site. The finding that the K154 residue is not conserved among all the organisms may indicate that the residue is not important for the function of the protein, or that TgBCP1 homologues lacking the K154 are not functional. Because *T. gondii* has more genetic tools available than *C. parvum*, it will likely be easier to analyze the importance of the lysine residue in *T. gondii* than *C. parvum*.

Other residues near the pocket are also conserved and may be involved in accessibility of the pocket. Specifically, PMxPITx(L/M)RNxLxxEGGSG(v/t) is highly conserved in family members (line on Fig. 3) and forms an alpha helix at the base of the pocket. Isomerization of the two highly conserved prolines in the helix could regulate the position of the helix to open or close the pocket. The top of the helix has some flexibility due to conserved glycines in the motif EGGSG. Opening the potential binding pocket may allow for the docking and modification of small molecules or peptides by TgBCP1 family members. While most peptidylprolyl bonds are in the energetically favored *trans*-formation, about 5–7% are found in the *cis*-formation. Isomerization of the peptidylprolyl bond from the *trans*- to the *cis*-position is commonly used to regulate the conformation, location, and binding capabilities of proteins. *T. gondii* contains at least three cyclophilins with peptidylprolyl *cis-trans* isomerase activity (Bell *et al.*, 2006). Interestingly, one of the pathogenesis mutants from the *T. gondii* STM screen has an insertion in the promoter of cyclophilin 20, indicating the potential importance of peptidylprolyl isomerases as virulence factors (Frankel *et al.*, 2007). Further characterization of TgBCP1 and its potential interaction with *T. gondii* cyclophilins may help identify the function of a large number of proteins that contain this motif.

The crystal structure of TgBCP1 and its *C. parvum* ortholog reveal possible catalytic amino acids and a pocket for unknown substrate, both of which could be drug targets. TgBCP1 is conserved in all Apicomplexans and its transcripts are abundant during all lifecycle stages. In fact, TgBCP1 is conserved across many different types of microbes, but is not present in humans, highlighting that it may be a great target for development of new broad-spectrum antimicrobials. TgBCP1 is likely essential for the tachyzoite stages as the protein is still present in the 38C3 mutant and multiple attempts to generate a complete deletion of the gene were not successful. Future studies on this intriguing family of proteins will determine their functional contribution to other microbial lifecycles.

Experimental procedures

Parasite strains and tissue culture growth conditions

The Pru HXGPRT (Pru), 38C3, C3 and transfected clones were maintained on monolayers of human foreskin fibroblast cells (HFF) grown in Dulbecco's Modified Eagle Media (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, and 100 µg/ml of penicillin and streptomycin. Cells were maintained in 5% CO₂ at 37°C. To ensure parasites did not evolve tissue culture-specific characteristics, parasites were frozen in 10% DMSO, 25% FBS, and 65% DMEM and passaged fewer than 10 times between thawing and experiments.

For bradyzoite development, confluent HFFs on coverslips or in 25 cm² tissue culture flasks were infected with either 1×10⁵ or 4×10⁶ tachyzoites respectively, and the parasites were allowed to invade for 3 hours. After invasion, the medium was replaced with bradyzoite development medium (0.5% FBS, 0.5% penicillin-streptomycin, and 21mM HEPES in RPMI 1640 without bicarbonate pH 8.0). Parasites were grown in bradyzoite development medium for 5 days.

Acute infections and cyst burden quantification

Six to eight-week old CBA/J or C57BL/6 mice from JAX labs were used for all studies. For acute infection, mice were infected with either 4×10⁵ or 1×10⁶ (Fig. 1B and C) tachyzoites by intraperitoneal (i.p.) injection. Animals were monitored daily for clinical signs of disease (ruffled fur, hunched posture, paralysis, etc.) and were euthanized if moribund. For quantification of the cyst burden, mice were infected with 2×10⁴ tachyzoites for initial studies (Fig. 1A), or 2×10⁵ tachyzoites for complementation studies (Fig. 7A) by i.p. injection. Mice were sacrificed at 22 days postinfection, their brains were removed, and ground with a mortar and pestle in 1.3 ml of phosphate buffered saline (PBS). 250 µl of the homogenized brain was fixed with 3.0% formaldehyde for 20 min, quenched with 1 mM glycine for 5 min, and permeabilized and blocked in 0.2% Triton X-100 with 3.0% bovine serum albumin (BSA) for at least 30 min. Tissue cysts were then stained with fluorescein-labeled *Dolichos biflorus* agglutinin (Vector Labs, Burlingame, CA, USA) for 1 hour, and 3–5 µL samples were mounted and cyst numbers counted by fluorescence microscopy. Samples were blinded to ensure no counting bias. Differences in chronic cyst formation between strains were analyzed by student T test.

Characterization of the *TgBCP1* mRNA

For cDNA synthesis, RNA was isolated using Ultraspec (BIOTECX, Houston, TX, USA), treated with amplification grade DNase (Invitrogen, Carlsbad, CA, USA), followed by reverse transcription with the Superscript III RT kit (Invitrogen) using random hexamers following manufacturers' protocols. cDNA was amplified with primers 38C3-5 (5'-AATCTGCCTCTTCGTGTTG-3') and 38C3-55 (5'-TCGCCTGTGAATGGTCCG-3') to determine the location of the introns and exons. The resulting product was TA cloned into PCR2.1 (Invitrogen) and sequenced with M13F and M13R primers. To determine the 3' end of the mRNA, cDNA was created as above with a poly-T anchor primer in place of the random hexamers. 5' RACE cDNA was made with random hexamers. 5' and 3' RACE were performed as described by FirstChoice® RLM-RACE (Ambion, Austin, TX, USA) with Taq DNA polymerase (Invitrogen). 5' RACE was performed with the inner primer 38C3-13 (5'-GAAAGCAGCAAAAACCAAACACGA-3') and 38C3-36 (5'-TCTTGTCTGTCTCCGCTTGTCCT-3'). 3' RACE was performed with the outer primer 38C3-12 (5'-TCCGATGAGCTATGCGAAAGACG-3'), and inner primer 38C3-10 (5'-TGGCTCCGATTACAATGCTCAGAA-3').

Southern and northern analysis

RNA isolated from $\sim 2 \times 10^7$ parasites using Ultraspec (BIOTECX, Houston, TX, USA) was separated on 1% formaldehyde agarose gels. To denature the RNA before transfer, the gel was soaked for 20 minutes in 10 mM NaOH in $5 \times$ SSC. DNA was isolated using the TELT method (60), digested and separated on 0.8%–1% agarose gels. The DNA or RNA was transferred in $20 \times$ SSC to Nytran Super Charge membrane (Schleicher & Schuell, Germany), except that the denaturing RNA gel was transferred in 10 mM NaOH in $5 \times$ SSC. Blots were pre-hybridized in 50% formamide, 0.12M Na_2HPO_4 , 0.25M NaCl, 7% SDS, 250mM EDTA, then probed with radio-labeled DNA probes for 16 hours and washed under stringent conditions. The cDNA probe was made by amplification of the *TgBCP1* ORF from Pru cDNA using primers 38C3-65 and 38C3ORF cDNA Pac I-R (5'-AATTAATTCTACTCCAGGGACGACT-3').

Plasmid construction and *T. gondii* transformations

The Best Kozak:*TgBCP1*-HA construct was made by amplification of the *TgBCP1* gene from Pru cDNA with the 38C3-65 primer (5'-atgcattcgaaaccggagtctc-3') and 38C3HA primer (5'-TTAATTAACCCGCGTAGTCTGGGACGTCGTATGGGTAgccgcccTCAGGGACGACTCTTTGTC-3') using TAQ polymerase (Invitrogen). The 685 bp piece was cloned into pBC SK+DHFR3 (Rooney *et al.*, 2011) at the Pac I and Nsi I sites. Use of *DHFR* allowed us to select for stable integration of the plasmid with 1 μM pyrimethamine (Donald and Roos 1993). ENDO:*TgBCP1*-HA was created by amplification of the promoter and 5' UTR with primers 38C3 36 (5'-TCTTGTCTGTCTCCGCTTGTCCT-3') and 38C3-63 (5'-GGGCCCCGTGTCTGCGTTTCTCACCAT-3'), followed by ligation to the cDNA open reading frame using splicing by overlapping extension PCR (59) with primers 38C3HA or 38C3 ORF cDNA Pac I. The 3.3 kb piece was TA cloned into pCR2.1 (Invitrogen), sequenced, and cloned into the Apa I and Pac I sites of Best Kozak:*TgBCP1*-HA. 10 ng of linearized plasmid was electroporated into 10^7 38C3 parasites (conditions

described in Boothroyd *et al.*, 1997). Parasites were grown under selection with pyrimethamine for 2 rounds of 4–5 days each, then inoculated into 96 well plates containing pyrimethamine for 7 days. Parasites that created single plaques in the 96 wells plates were screened by Southern blot for unique banding patterns to ensure unique insertion events.

Western blot detection of TgBCP1

Western immunoblots were performed on reduced protein lysates from tissue culture tachyzoites and bradyzoites or bradyzoite cysts were purified with a Percoll gradient (Blewett *et al.*, 1983). To ensure proteins from the parasitophorous vacuole and membrane were included in the sample, tissue culture lysates were generated from intracellular parasites and the monolayer was scraped but not syringed. Proteins were separated on 10% polyacrylamide gels, transferred to Hybond nitrocellulose membranes (Amersham/GE Healthcare Life Sciences, Pittsburgh, PA, USA) and the membranes probed with primary antibodies 1:1000 mouse anti-HA or 1:300 mouse polyclonal antiserum developed against TgBCP1 as previously described (Harlow and Lane, 1988) with TgBCP1 protein generously supplied by the Anderson lab. Secondary antibody was 1:5000 affinity-purified horseradish peroxidase (HRP) conjugated donkey anti-mouse antibodies (Jackson Immuno Research West Grove, PA, USA) and signals were detected using ECL Plus Western Detection solution (Amersham/GE Healthcare Life Sciences).

Immunofluorescence assay

Immunofluorescence assays were carried out with parasites grown on coverslips in HFF cells for 24 hours, or mice for 28 days. Infected HFFs or brain lysates were washed with PBS, fixed with formaldehyde for 20 min at room temperature, and quenched with 1 mM glycine. Fixed parasites were blocked and permeabilized with 3% BSA/0.2% TritonX100 for one hour at room temperature or over night at 4°C. Tachyzoites were incubated with 1:300 mouse polyclonal antiserum developed against TgBCP1 and either 1:500 rabbit anti-GRA2 or 1:300 rabbit anti-NHE3. For tachyzoites containing HA-complementation constructs, cells were incubated with 1:2000 HA.11 clone 16B12 mouse anti-HA (Covance, Princeton, NJ, USA) and 1:300 rabbit anti-HXGPRT antibody. Secondary antibodies were AlexaFluor 488 goat anti-mouse antibody and AlexaFluor 633 conjugated goat anti-rabbit (Invitrogen). For bradyzoites, cells were incubated in 1:100 mouse anti-TgBCP1 antisera or 1:500 rabbit anti-HA (Abcam ab9110) and either 1:500 rabbit anti-BAG1 or 1:500 Biotinylated Dolichos Biflorus Agglutinin (Vector Laboratories, Burlingame, CA, USA). Secondary antibodies AlexaFluor 488 goat anti-mouse or goat anti-rabbit and AlexaFluor 633 conjugated to goat anti-rabbit or to streptavidin. All IFAs were examined with a Zeiss Axioplan Ili microscope equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, differential interference contrast (DIC) optics, and a Hamamatsu ORCA-AG CCD camera. Images were acquired at 100× (oil immersion) in three channels with a 0.2 micron Z-step series. Openlab version 4.1 (PerkinElmer, Waltham, MA, USA.) was used to deconvolve and pseudocolor the images with a constrained iterative algorithm. Individual images are presented as a summed stack image for each channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We sincerely thank Wayne Anderson for purified TgBCP1 protein for antibody production, Christina Hull, Wayne Anderson and Jon Boyle for helpful discussions, Michael Wollenberg for assistance with phylogenetic analysis, Will Olson and Patrick Cervantes for percoll purified brain cysts, and Jay Bangs and Margaret McFall-Ngai for use of the Zeiss Axioplan Ili microscope. For antibodies we thank David Sibley for GRA2 and β -tubulin, Louis Weiss from BAG1, and Gustavo Arrizabalaga for NHE3. Research for this project was conducted with government support under contract FA9550-11-C-0028 and awarded by the Department of Defense, Air Force office of Scientific Research, National Defense Science and Engineering Graduate (NDSEG) Fellowship, 32 CFR 168a (S.K.W.), and by the National Institutes of Health (NIH) National Research Service Award GM072125 (K.M.M.), NIH R21AI114277 and R03AI077345 (L.J.K.).

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Fig. 1. 38C3 mutant has a reduced number of brain cysts but similar acute virulence

A. Mice were infected with 2×10^4 parasites of either Pru , 38C3 or C3 strains. The Y-axis shows the number of cysts per mouse brain 22 days post infection. Each symbol represents brain cysts from an individual mouse infected with the strain shown on the X axis. Four mice per strain. Horizontal bar indicates average cysts formed by each strain. There was a statistically significant difference between Pru and 38C3 ($p < 0.05$), between Pru and C3 ($p < 0.05$), and between 38C3 and C3 ($p < 0.01$) using one-way ANOVA with post-hoc Tukey.

One of the two independent experiments is represented by this graph; both had similar results.

B and C. Mice were infected with either 4×10^5 (panel B, solid lines) or 1×10^6 (panel C, dashed lines) of either Pru , 38C3 or C3 parasites. The Y-axis shows the percent survival and the X-axis days post infection. Data is combined from a total of 14 mice in four independent experiments for Pru , and 18 mice from five independent experiments for 38C3 and C3 strains. By the long-rank method, for panel B, $p < 0.0001$ for both Pru vs. C3 and Pru vs. 38C3. For panel C, $p < 0.0001$ for Pru vs. C3 and $p = 0.0003$ for Pru vs. 38C3. For 38C3 vs. C3, $p = 0.5$ for both panel B and C.

Fig 2. 38C3 mutant has an insertion in the TgBCP1 gene that disrupts the mRNA

A. The ends of the mRNA of the disrupted gene in 38C3 were mapped by 5' and 3' RACE using tachyzoite RNA. Triangle indicates the STM insertion site was in between the first two potential start codons (1st ATG, 2nd ATG). The third in frame start codon has the optimal *T. gondii* consensus sequence (BK ATG). The six introns (white boxes) and the seven exons (black boxes) were mapped by sequencing mature cDNA; 5' and 3' UTRs (dotted boxes) determined from the Best Kozak ORF.

B. Northern blot analysis of RNA collected from either Pru Δ , mutant 38C3 or parental C3 strains. The membrane was probed with TgBCP1 cDNA (top), then stripped and re-probed with the alpha-tubulin (α TUB) as a loading control (bottom). Numbers to the left of the membrane indicate RNA ladder in kilobase pairs.

C. Northern blot analysis of RNA collected from either parental C3 or mutant 38C3 strains. The gel was treated with NaOH to denature the mRNA before and during transfer. The membrane was probed with TgBCP1 cDNA. Numbers to the left of the membrane indicate RNA ladder in kilobase pairs. Arrows on the side indicate the bands.

Clustal Omega (Sievers *et al.*, 2011) was used to align TgBCP1 and four orthologs from fellow Apicomplexans. The three potential start codons are marked with arrows. The black line over the amino acids marks the domain conserved between all family members. * indicates identical amino acids between all five family members, a single dot indicates conservation in most family members, and double dots indicate similar side chains in all five family members.

Fig. 4. Translation of TgBCP1 starts from the third in frame start codon and still occurs in 38C3
A. Endo:TgBCP1-HA is TgBCP1 expressed from the endogenous promoter with the native 5'UTR (dotted box), ORF amplified from cDNA (black box), carboxyl-terminal HA tag (diamond shape) and SAG1 3'UTR (striped box). First (1st), second (2nd) and Best Kozak (BK) ATGs are marked on the 5'UTR.
B. Best Kozak:TgBCP1-HA is TgBCP1 expressed from the α -tubulin promoter with the α -tubulin 5'UTR (horizontal striped box), ORF amplified from cDNA (black box), carboxyl-terminal HA tag (diamond shape) and SAG1 3'UTR (striped box).

C. Western blot analysis of parasites containing either Best Kozak:TgBCP1-HA (BK), no construct (Pru) or Endo:TgBCP1-HA (Endo). Incubation with anti-HA antibody showed that TgBCP1 expressed from the endogenous promoter and 5'UTR is the same size as TgBCP1 expressed exclusively from the third in frame methionine. Numbers to the right of the membrane indicate protein marker sizes in kilodaltons.

D. Western blot analysis of C3, 38C3, two independent clones of 38C3 transfected with Endo:TgBCP1-HA (Endo1 and Endo2) or two independent clones of 38C3 transfected with Best Kozak:TgBCP1-HA (BK1 and BK2). Parasites were grown in either tachyzoite (Tachy) or bradyzoite (Brady) conditions in tissue culture. Upper blot was incubated with anti-TgBCP1 antibody (TgBCP1), the middle blot was incubated with anti-HA antibody (HA) and the lower blot was incubated with anti-*T. gondii* β -tubulin as a loading control (β -TUB). The numbers to the left of the membrane indicate protein marker sizes in kilodaltons. Because of the over expression of TgBCP1-HA in the BK1 and BK2 lanes, one quarter the amount of protein was loaded per lane.

Fig 5. TgBCP1 has punctate localization within tissue culture tachyzoite and bradyzoite parasites C3 and 38C3 parasites were grown for either 36 hours in tachyzoite or 5 days in bradyzoite conditions. Fixed and permeabilized cells were incubated with anti-TgBCP1 antibody (green) and for the red channel either dense granule protein 2 (GRA2) or Na(+)/H(+) exchanger (NHE3) for tachyzoites and Dolichos biflorus agglutinin (DBA) or bradyzoite heat-shock protein (BAG) for bradyzoites. Cells were mounted with DNA binding 4–6'diamidino-2-phenylindole (DAPI) to stain the nucleus of the parasites and host cells blue and imaged using differential interference contrast (DIC).

Fig. 6. Complementations of the 38C3 mutant with TgBCP1 and size of TgBCP1 in brain cysts
A. Mice were infected with 2×10^5 parasites of either C3, 38C3, two independent clones of 38C3 transfected with Endo:TgBCP1-HA (Endo1 and Endo2) or two independent clones of 38C3 transfected with Best Kozak:TgBCP1-HA (BK1 and BK2). The Y axis shows the number of cysts per mouse brain 22 days post infection. Each symbol represents brain cysts from an individual mouse infected with the strain shown on the X axis. For C3, 38C3, Endo1 and Endo2, shown are the combined results from two independent experiments of 6 mice each, 12 mice total. For BK1 and BK2, shown are the results from one experiment with 6 mice each. Two C3 and one Endo1 infected mice became moribund and needed to be sacrificed during the experiment on days 18, 20 and 16 respectively. Using an independent T-test without equal variance, statistically significant difference between C3 and 38C3 ($p < 0.0001$), 38C3 and Endo1 ($p < 0.0001$), 38C3 and Endo2 ($p < 0.005$), and between C3 and BK1 and BK2 ($P = 0.003$ for both).
B. Western blot analysis shows lysates of *Pr* parasites grown in tissue culture conditions as tachyzoites (Tachy, lane 1) or percoll purified brain cysts from mice infected with 2×10^4 *Pr* parasites for 28 days (Brain, lane 2). Numbers to the right of the membrane indicate protein marker sizes in kilodaltons.

Fig. 7. TgBCP1 is localized to the parasitophorous vacuole space and cyst wall in brain cysts
Mice were infected with 2×10^4 Pru parasites for 28 days, then their brain homogenized and stained for TgBCP1 (green) with the anti-TgBPC1 antibody or anti-HA antibody, DBA as a marker of the cyst wall (red) or BAG1 as a marker of the bradyzoite cytoplasm (red), and imaged using differential interference contrast (DIC). All images were photographed at the same magnification, with the yellow scale bar at 5 μ m.