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Targeted Disruption of Toxoplasma gondii Serine Protease Inhibitor 1 Increases Bradyzoite Cyst Formation *In Vitro* and Parasite Tissue Burden in Mice

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**Abstract:** As an intracellular protozoan parasite, Toxoplasma gondii is likely to exploit proteases for host cell invasion, acquisition of nutrients, avoidance of host protective responses, escape from the parasitophorous vacuole, differentiation, and other activities. T. gondii serine protease inhibitor 1 (*TgPI1*) is the most abundantly expressed protease inhibitor in parasite tachyzoites. We show here that alternative splicing produces two *TgPI1* isoforms, both of which are secreted via dense granules into the parasitophorous vacuole shortly after invasion, become progressively more abundant over the course of the infectious cycle, and can be detected in the infected host cell cytoplasm. To investigate *TgPI1* function, the endogenous genomic locus was disrupted in the RH strain background. Δ*TgPI1* parasites replicate normally as tachyzoites but exhibit increased bradyzoite gene transcription and labeling of vacuoles with Dolichos biflorus lectin under conditions promoting *in vitro* differentiation. The differentiation phenotype can be partially complemented by either *TgPI1* isoform. Mice infected with the Δ*TgPI1* mutant display ~3-fold-increased parasite burden in the spleen and liver, and this *in vivo* phenotype is also complemented by either *TgPI1* isoform. These results demonstrate that *TgPI1* influences both parasite virulence and bradyzoite differentiation, presumably by inhibiting parasite and/or host serine proteases.
Introduction

Parasitic life history strategies might be expected to balance host-pathogen interactions so that the competition between virulence factors and immune responses produces an equilibrium ensuring the survival of both the parasites and their hosts (2). Proteases have been shown to play an important role in pathogenesis in many viral, bacterial, and parasitic systems (20, 24, 33), and the regulation of protease activity is likely to be a critical aspect of pathogen biology (1). Serine protease inhibitors are commonly grouped into categories based on their primary sequences, structural motifs, and mechanisms of binding (28), and the Kazal, Kunitz, Serpin, and Smapin (small serine protein inhibitor) families have all been implicated in pathogen survival (21, 31, 32, 49, 51, 55, 56).

Protease inhibitors have been extensively characterized in metazoa but are absent from most of the protozoan taxa for which complete genomes are available (10). However, putative Kazal-type inhibitors have been identified in a subclass of apicomplexan parasites termed the Coccidia including Cryptosporidium (four genes), Neospora (at least two), and Toxoplasma (six); noncoccidial apicomplexan parasites (Plasmodium, Babesia, Theileria, etc.) show no evidence of any serine protease inhibitors. While the functions of coccidial Kazal-type protease inhibitors are not known, they have been hypothesized to protect parasites from proteolytic damage in the gut, suppress proteolytic activity during parasite replication, and counteract host proteases of the innate immune system (8, 39, 40, 44). Kazal-type protease inhibitors consist of one or more domains, each containing six conserved cysteines that form three intradomain disulfide bonds. Each domain displays an accessible surface loop containing a peptide bond called the reactive site, which specifically interacts with the active site of the target protease (44). All coccidian Kazal-type inhibitors appear to be “nonclassical” based on the short spacing between cysteines 1 and 2, a property that is thought to impart greater specificity for the target protease (22).

Biochemical studies have shown that Toxoplasma gondii serine protease inhibitor 1 (TgPI1) inhibits a broad range of serine proteases (40), while TgPI2 inhibits trypsin (39) and Neospora caninum PIS (NcPIS) inhibits subtilisin (8, 41). All appear to traffic via the default “dense-granule” secretory pathway into the parasitophorous vacuole (PV), within which these obligate intracellular parasites replicate (26). Apicomplexan Kazal-type inhibitors could potentially target either parasite or host proteases, but their physiologically relevant targets remain unknown. To investigate the functions of these inhibitors, we genetically deleted TgPI1, the dominant Kazal inhibitor in T. gondii (expressed at least 10-fold more highly than TgPI2), which is present as two isoforms during all major stages of the parasite life cycle (tachyzoites, bradyzoites, and sporozoites). ΔTgPI1 mutants exhibit altered differentiation and in vivo growth phenotypes that can be complemented by either TgPI1 isoform.

Materials and Methods

Parasite and cell culture. All parasite strains were propagated as tachyzoites in human foreskin fibroblasts (HFF), as previously described (47). Wild-type RH, Prugniaud, and VEG strains were used as representatives of the type I, II, and III lineages defined by population genetic studies (23). Mutant parasites were constructed in the RHΔHXGPRT knockout background (16).
**Western blotting, metabolic labeling, and immunoprecipitation.** Parasites were harvested from infected HFF (in T25 flasks) by scraping and passage through a blunt 27-gauge needle, and the centrifuged pellets were resuspended in pH 7.6 phosphate-buffered saline (PBS) containing 5 mM MgCl₂, 0.5% Triton X-100, 100 U/ml DNase, and protease inhibitors (Sigma P8340). Proteins (∼10⁷ tachyzoite equivalents per lane) were analyzed by SDS-PAGE on Novex bis-tris 4 to 12% gradient gels (Invitrogen) in parallel with prestained standards. After electrotransfer to nitrocellulose, the membranes were blocked in PBS containing 5% nonfat dry milk and 0.05% Tween 20 prior to adding rabbit anti-\( Tg\)PI1 (1:5,000) (44). After washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) and visualized by chemiluminescence (GE Healthcare).

For metabolic labeling, confluent HFF monolayers in T175 flasks were infected with ∼4 × 10⁷ tachyzoites 20 h before labeling for 15 min with \[^{35}\text{S}]\text{methionine/cysteine} (50 mCi/ml). After washing, the cultures were chased for 0, 10, 25, or 60 min in unlabeled medium; harvested by scraping and centrifugation; passed 3 times through a 25-gauge needle; and solubilized in 1 ml RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 100 mM NaCl, 5 mM EDTA) containing 10 mg/ml RNase A, 20 mg/ml DNase I, and protease inhibitors. Samples were preincubated overnight at 4°C with protein G-Sepharose alone and centrifuged, and the supernatants were incubated for 1 h in rabbit anti-\( Tg\)PI1 antiserum, followed by the addition of protein G-Sepharose and further incubation for 1 h. The precipitated complexes were washed 4 times, boiled in electrophoresis buffer containing 10% SDS and 2% β-mercaptoethanol, separated by polyacrylamide gel electrophoresis, incubated in Amplify fluorographic enhancer (Amersham), dried on cellophane, and exposed to X-ray film.

**MALDI mass spectrometry.** Matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager DE-STR (PerSeptive Biosystem) equipped with a 337-nm nitrogen laser. Unless otherwise specified, spectra were obtained in positive acquisition mode, and external peptides were used for calibration; the reported peptide masses are monoisotopic, while intact protein masses are average masses.

Parasites were cultivated in five T175 flasks until complete lysis of the host cell monolayer occurred, centrifuged, and passed through a 0.45-µm filter, and the filtrate (containing secreted \( Tg\)PI1) was incubated overnight with protein A-agarose beads (Bio-Rad) coupled to polyclonal rabbit anti-\( Tg\)PI1. After extensive washing with Tris, pH 8.0, bound protein was eluted in 100 mM glycine, pH 3.0, and protein-containing fractions (Bradford assay) were pooled and concentrated in a Centricon filter (Millipore). After electrophoresis on polyacrylamide gels and staining with copper, intact proteins were excised and extracted using standard methods (11): bands were sequentially incubated in 50 mM NH₄HCO₃ (10 min), followed by 50% acetonitrile, 200 mM NH₄HCO₃ (30 min, twice), and then high-performance liquid chromatography (HPLC) grade water; crushed with a pointed dental tool; covered with 20 μl of 10 mg/ml sinapinic acid (matrix) in 0.5% trifluoroacetic acid (TFA) in acetonitrile; and extracted by vortexing for 3 h at room temperature. Following extraction, the tubes were opened to evaporate the contents for ∼20 min with vortexing until the matrix solution became slightly cloudy, and 1 μl supernatant was deposited onto the MALDI probe. After calibration with aldolase, spectra were acquired in linear mode (acceleration, 25 keV; 91% grid voltage; 1,000-ns delay; 100 laser shots per
spectrum), yielding an error of <10 atomic mass units. Peak masses were assigned based on the centroid at 50% height, and standard deviations were calculated based on at least three independent spectra per protein.

For peptide analysis, bands were excised, reduced by 1 h of incubation at 56°C in 50 mM dithiothreitol (in 100 mM NH₄CO₃), and alkylated by treatment for 45 min with 275 mM iodoacetamide (in 100 mM NH₄CO₃) in the dark. After in-gel digestion with 12.5 ng/μl trypsin in 50 mM NH₄HCO₃ and solvent extraction (50), the peptide mixtures were dried, dissolved in 2 μl 50% acetonitrile plus 0.3% TFA, mixed with saturated CHCA (α-cyano-4-hydroxycinnamic acid) matrix, and deposited on the MALDI MS target plate. Spectra were acquired in reflection mode with delayed extraction. Peptide mass fingerprints were used to search against the NCBI nonredundant database using MS-Fit (http://prospector.ucsf.edu) or FindPept (http://web.expasy.org/findpept/), based on a threshold mass deviation of 50 ppm.

**Molecular genetic manipulations.** Total parasite RNA was isolated from freshly harvested tachyzoites (RNAEasy; Qiagen), and 2 μg purified RNA was subjected to reverse transcription (RT)-PCR (60 min of reverse transcription at 42°C using Moloney murine leukemia virus reverse transcriptase [Promega]) amplification using Expand High Fidelity PCR (Roche) and primers 5′-GATCGGATCCGCTCTGCGCCGAAACGAAA-3′ (forward; BamHI site is underlined) and 5′-GATCGGTACCCTGGTGATCATCCCAGATCCTCTCG-3′ (reverse; KpnI site is underlined). PCR product sizes were analyzed by gel electrophoresis, and the products were sequenced.

The TgPI1 targeting construct was engineered by PCR amplifying 3.6 kb of TgPI1 3′ flanking sequence with primers 5′-GATCGGATCCGCTCTGCGCCGAAACGAAA-3′ (forward; BamHI site is underlined) and 5′-GATCGAGCTCTGGTGATCATCCCAGATCCTCTCG-3′ (reverse; SacI site is underlined) and cloning it downstream of the dhfrHXGPRTdhfr selectable marker (12). 5′ TgPI1 sequence (2.5 kb) was amplified using primers 5′-GATGGTGTAGTTGAGGCTGATTTGC-3′ (forward) and 5′-AGATCTGGGCCCTAAGCTTTACGACCGGTAGCAC-3′ (reverse; underlined sequence indicates a link added to facilitate further fusion PCR) and joined to the dhfrHXGPRTdhfr cassette plus 3′ TgPI1 sequences amplified using primers 5′-AGCTTATGGGCCCTAAGCTTTACGACCGGTAGCAC-3′ (forward; link sequence is underlined) and 5′-CCTCTAAGCTTTACGACCGGTAGCAC-3′ (reverse; underlined sequence indicates a link added to facilitate further fusion PCR) and 5′-GATGGTGTAGTTGAGGCTGATTTGC-3′ (forward; link sequence is underlined) and 5′-GATCGGATCCGCTCTGCGCCGAAACGAAA-3′ (forward; BamHI site is underlined) and 5′-GATCGGTACCCTGGTGATCATCCCAGATCCTCTCG-3′ (reverse; KpnI site is underlined). PCR product sizes were analyzed by gel electrophoresis, and the products were sequenced.

RHΔHXGPRT strain parasite tachyzoites (10⁷) (16) were directly transfected with 30 μg of the 8.1-kb fusion PCR product and selected in 25 μg/ml mycophenolic acid plus 50 μg/ml xanthine as previously described (16).

Surviving populations were screened for (i) loss of the TgPI1 locus using primers 5′-GATCGGATCCGCTCTGCGCCGAAACGAAAATCCG-3′ (P1) (forward; BamHI site is underlined) and 5′-GATCGGATCCGCTCTGCGCCGAAACGAAAATCCGCC-3′ (P2) (reverse; AvrII site is underlined), (ii) presence of the HXGPRT selectable marker using primers 5′-GATCGGATCCGCTCTGCGCCGAAACGAAAATCCGCC-3′ (P1) and 5′-GATCGGATCCGCTCTGCGCCGAAACGAAAATCCGCC-3′ (P2), and (iii) homologous recombination of HXGPRT into the TgPI1 locus using primers P3 and 5′-
CTAGATGCAGTCTCGGAGATAGCTCAT-3' (P5). Positive populations were cloned by serial dilution in 96-well plates, and the isolated clones were screened using the same primers.

Fluorescent fusion proteins (for subcellular localization) and glutathione S-transferase (GST) fusion proteins (for functional complementation and purification) were engineered by amplifying TgPI1α or TgPI1β coding sequences using primers 5′-GATCGGATCCGGGATACCCCTTTCGAGATCT-3′ (forward) and 5′-GATCCCTAGGTGTTGGTACACGATCTCTTCGG-3′ (reverse) and cloning into plasmid tubmRFP, tubYFP, or tubGST (42), all of which harbor a sagCATsag cassette; selected in 20 μM chloramphenicol; and cloned by limiting dilution.

For genomic hybridization, 5 μg DNA from parental RHΔHXGPRT parasites, TgPI1 knockout mutants, and complemented clones was digested with EcoRI, separated on a 0.7% agarose gel, transferred to a Hybond N+ membrane (Amersham), and hybridized with digoxigenin probes (Roche) generated by PCR amplification of coding sequence primers 5′-CGCAGCAGGATGACGAATCTGA-3′ (forward) and P2 (see above) or 5′ flanking region primers 5′-CTCCTGTACCCCTTACGACTTCGTC-3′ (forward) and 5′-CGTCCAGTTTCTCGTGCCTGAAT-3′ (reverse).

For expression profiling, confluent monolayers of HFF grown in T25 flasks containing Dulbecco's modified Eagle medium supplemented with 20% medium 199 (Life Technologies) and 10% cosmic calf serum (HyClone) were inoculated with 5 × 10⁵ RHΔHXGPRT or ΔTgPI1 tachyzoites, and RNA was extracted after 36 h (RNeasy kit; Qiagen). The RNA was labeled using Ovation Amp v2 (NuGen) according to the manufacturer's instructions and hybridized to a custom T. gondii Affymetrix array (3). Gene expression levels were determined using robust multichip average (RMA) normalization.

**Fluorescence microscopy.** HFF grown on glass coverslips and infected with parasites were fixed for 20 min in 4% paraformaldehyde plus 0.02% glutaraldehyde, permeabilized for 15 min in 0.2% Triton X-100, washed several times in PBS, blocked for 30 to 60 min in 5% fetal bovine serum plus 3% bovine serum albumin (fraction V; Sigma), and incubated for 1 h with primary antibody (see above), followed by 1 h of incubation with Alexa 594- or Alexa 488-conjugated goat anti-mouse or goat anti-rabbit antiserum (Molecular Probes). The slides were mounted in Fluormount G (Southern Biotechnology) and imaged at ×100 using a Leica DM IRB microscope equipped with a high-resolution charge-coupled-device (CCD) camera (Orca-ER; Hamamatsu) and Openlab 5.5.1 software (Improvision).

**In vitro assays:** parasite replication and differentiation. Intracellular T. gondii replication was assessed as previously described (19) by inoculating confluent HFF monolayers in six-well plates with 2 × 10⁵ freshly purified tachyzoites. The monolayers were washed to remove extracellular parasites at 4 h, and replication was assessed at 12, 24, 30, and 36 h by fixation in 3.7% paraformaldehyde, staining with Giemsa stain, and scoring the number of intracellular parasites in 100 parasitophorous vacuoles selected at random. Each time point represents triplicate assays from two independent experiments; statistical analysis was based on the two-tailed Student's t test.
Parasite differentiation (17) was induced by inoculation of HFF monolayers in six-well plates, switching to minimal essential medium (MEM) containing 1 g/liter NaHCO₃ plus 50 mM Tricine (pH 8.1) at 4 h postinfection, and incubation at 37°C in 0.03% CO₂, replacing the medium every 6 to 8 h to maintain a constant alkaline pH. At 48 h, the cultures were fixed in 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked with 10% fetal bovine serum, and stained with 1:100 tetramethyl rhodamine isocyanate (TRITC)-conjugated Dolichos biflorus lectin (Sigma). One hundred randomly selected parasitophorous vacuoles were scored for a homogeneous cyst wall (average of three independent experiments).

**Quantification of the parasite burden in vivo.** Female 6- to 8-week-old BALB/c mice were infected by intraperitoneal injection of 10³ parasite tachyzoites (RH wild type, ΔTgPI1 mutant, or RHΔTgPI1 complemented with TgPI1α or TgPI1β). After sacrifice at 7 days postinfection, DNA was extracted from the liver, spleen, and brain (High Pure PCR Kit; Roche), and 100 ng of each sample was analyzed by quantitative PCR (25) using primers 5’-TCTTTAAAGCGTTCGTGGTC-3’ (forward) and 5’-GGAACTGCATCCGTTCATGAG-3’ (reverse) to determine the abundance of the multicopy T. gondii B1 gene (25) relative to a mouse β-actin control (forward, 5’-TCACCCACACTGTGCCCATCTACGA-3’; reverse, 5’-CAGCGGAACCGCTCATTGCCAATGG-3’). After 10 min of denaturation at 95°C, thermal cycling was carried out using 40 cycles of 1 min annealing/extension at 60°C, alternating with 15 s of denaturation at 95°C (Applied Biosystems 7500) and SYBR green detection. The 2⁻ΔΔCT method was used to calculate the relative parasite burdens in different tissues; analysis of serial dilutions of DNA from infected livers demonstrated similar amplification efficiencies for the TgB1 target and mouse β-actin over a wide range of DNA concentrations.

**Nucleotide sequence accession numbers.** Updated TgPI1 sequence information is available at ToxoDB.org (TgME49_008450) and in GenBank (accession numbers HM536610 [TgPI1α], AF121778 [TgPI1β], and HM536941 [genomic]).

**Results and Discussion**

Alternative splicing of a 72-nucleotide exon produced two TgPI1 isoforms. Immunoblotting demonstrated two TgPI1 isoforms in each of the three dominant lineages of T. gondii (Fig. 1A), as previously described for RH strain parasites (40, 44). Expression levels of the smaller isoform (designated TgPI1β) were comparable in all strains tested, but the larger isoform (TgPI1α) was reproducibly less abundant than TgPI1β in strain RH, more abundant than TgPI1β in the Pru strain, and comparable to TgPI1β in VEG strain parasites.

To determine whether these two isoforms arise by posttranslational modification, extracellular T. gondii strain RH tachyzoites were labeled with ³⁵S-labeled amino acids for 15 min, and TgPI1 was immunoprecipitated after 0 to 60 min of incubation in unlabeled medium. Both isoforms were observed at all time points (Fig. 1B), indicating that TgPI1α and -β do not exhibit a precursor-product relationship, i.e., they are probably not attributable to proteolytic cleavage or other posttranslational modifications. Also, the altered migration is not due to differential N-linked glycosylation occurring cotranslationally in the endoplasmic reticulum, as the TgPI1 deduced sequence is devoid of a consensus motif (NXS/T) for N-glycan addition.
To further investigate the relationship between \( TgPI1\alpha \) and \( -\beta \), the isoforms were immunoaffinity purified from the supernatant of a heavily infected HFF monolayer shortly after parasite egress (when \( TgPI1 \) is released from the disrupted vacuoles), and bands obtained by gel electrophoresis were subjected to MALDI-TOF mass spectrometry (Fig. 1C). Intact protein masses were measured at 34.3 kDa for \( TgPI1\alpha \) and 31.5 kDa for \( TgPI1\beta \), and the molecular mass difference between the isoforms was estimated to be 2,775 ± 31 Da. A peptide mass fingerprint generated by tryptic digestion prior to MALDI-TOF revealed very similar spectra for \( TgPI1\alpha \) and \( TgPI1\beta \) (Fig. 1D), differing only in the presence of a unique \( m/z \) peak at 1,652.7. This peak corresponds to a 15-amino-acid tryptic peptide (TQSSHQHDDAENGAR) that is unique to \( TgPI1\alpha \), suggesting that \( TgPI1\alpha \) has a distinct genetically encoded segment.

To determine if the distinct segment is due to differential RNA splicing, we performed RT-PCR of total RNA from RH strain parasites using forward and reverse primers encompassing the full-length \( TgPI1 \) coding sequence. This analysis yielded distinct transcripts of 887 nucleotides (nt) and 815 nt; the same bands were obtained by RT-PCR from an RH strain tachyzoite cDNA library, while a single band of 2,973 nt was obtained from genomic DNA (Fig. 1E). The relative abundances of the two \( TgPI1 \) transcripts correlate with the relative abundances of the two protein isoforms in RH strain parasites (Fig. 1A, lane 1). Sequencing of the cloned products revealed that the larger species contains a 75-nt insertion predicted to encode 25 amino acids, including the extra peptide identified by mass spectrometry. This 25-amino-acid insert has a predicted mass of 2,752 Da, which is within the measurement range of the mass difference between \( TgPI1\alpha \) and \( TgPI1\beta \) (2,775 ± 31 Da). Comparison with the \( TgME49_008450 \) genomic locus (ToxoDB.org) identified the extra sequence as a distinct exon, defining alternatively spliced mRNAs of 8 versus 9 exons (Fig. 1F); several expressed sequence tags (ESTs) validate each of these transcripts and confirm the alternative splicing of \( TgPI1 \). All predicted \( TgPI1 \) introns begin with GT and end with AG, in agreement with the consensus for other eukaryotes (including \( T. gondii \)), and sequence upstream of the first ATG corresponds to the consensus for translational initiation in \( T. gondii \) (AAAATG). The first 24 amino acids (most of the first exon) are predicted to comprise a secretory signal sequence. Exons 2, 4, 7, and 9 each encode a Kazal domain (asterisks in Fig. 1F), while exons 1, 3, 5, 6, and 8 encode linkers between the Kazal domains (44); the alternatively spliced exon expands the linker between the third and fourth Kazal domains in \( TgPI1\alpha \). This modular structure is consistent with the repeated duplications typical of multidomain Kazal inhibitor evolution (29). The identification of alternative splicing of the \( TgPI1 \) gene adds to the growing list of examples of transcript (and protein) diversity generated by splicing in \( T. gondii \) (14, 16, 30, 53).

Targeted deletion and complementation of the \( TgPI1 \) locus. To investigate the function of \( TgPI1 \), the genomic locus was deleted by homologous replacement using HXGPRT as a positive selectable marker in the \( RH\DeltaHXGPRT \) background (16). Figure 2A displays the \( TgPI1 \) genomic locus and the targeting construct used to generate \( \Delta TgPI1 \) knockout parasites. Transfected parasites were inoculated into HFF in six wells of a 24-well plate, selected in mycophenolic acid, and screened by PCR upon host cell lysis (~5 to 7 days posttransfection) to identify wells containing candidate knockouts. Individual parasites from two positive wells were cloned by limiting dilution in 96-well plates and rescreened by PCR (Fig. 2B) to identify clones lacking the \( TgPI1 \) gene (Fig. 2A, P1-P2) in which the selectable marker had integrated at the endogenous
TGPI1 locus (P3-P4 and P3-P5). Gene replacement was confirmed by Southern blotting: a probe amplified from genomic DNA showed complete loss of TGPI1 coding sequence (Fig. 2C, left, compare lane 1 with lanes 2 and 3), and a flanking sequence probe demonstrated the expected 800-nt decrease in size from the 12.3-kb EcoRI fragment encompassing TGPI1 (Fig. 2C, right, lane 1 versus lanes 2 and 3).

For genetic complementation, TGPI1 knockout (ΔTGPI1) parasites were transfected with plasmids engineered to express cDNA encoding TGPI1α or TGPI1β fused to GST, to facilitate future pull-down studies, flanked by a β-tubulin promoter and a dihydrofolate reductase-thymidylate synthase (DHFR-TS) 3′ untranslated region. A chloramphenicol acetyltransferase (CAT) selectable marker was incorporated into the complementation plasmid, and Southern blotting of genomic DNA from clonal parasite transfectants revealed the expected 5.2-kb EcoRI fragment containing the TGPI1 transgene; additional bands corresponded to integration events fragmenting the TGPI1 transgene (Fig. 2C, left, lanes 4 and 5). Western blotting using rabbit anti-TGPI1 (Fig. 2D) or anti-GST (not shown) demonstrated loss of TGPI1 in ΔTGPI1 parasites and expression of a single TGPI1-GST protein of the expected size in TGPI1α or TGPI1β transgenic parasite lysates (smaller bands may be attributable to proteolytic cleavage).

Targeted deletion of a gene family member in T. gondii can lead to upregulation of another member of the gene family (46). To determine whether deletion of TGPI1 resulted in compensatory upregulation of other T. gondii genes, including those encoding other serine protease inhibitors, we performed genome-wide expression profiling using a custom T. gondii Affymetrix microarray (Toxodb.org) (Fig. 2E). Interpro motif searches identified eight putative protease inhibitors in the T. gondii genome: six Kazal-type inhibitors (TGME49_008450 [TGPI1], TGME49_008430 [TGPI2], TGME49_083470, TGME49_059890, TGME49_024080, and TGME49_066610) and two serpins (TGME49_017430 and TGME49_046130). Only four of them exhibited steady-state transcript levels above background (TGPI1, TGPI2, and the two serpins). As expected, TGPI1 transcripts were reduced to background levels (>200-fold decrease) in the ΔTGPI1 mutants. Steady-state transcript levels for TGPI2 were ~10-fold lower than for TGPI1 in both control parasites and the ΔTGPI1 knockout. This finding confirms earlier studies showing low but detectable expression of TGPI2 protein in tachyzoites (39), and it demonstrates a lack of compensation by TGPI2 in the ΔTGPI1 knockout. Similarly, no compensatory expression or other changes were observed in other protease inhibitor genes. These observations were confirmed by quantitative PCR (not shown).

TGPI1α and TGPI1β are secreted into the parasitophorous vacuole and are also transferred to the host cell cytoplasm. Indirect immunofluorescence using rabbit antisera to recombinant TGPI1 showed expression of TGPI1 in parental RHΔHXGPRT parasites (Fig. 3A), loss of expression in ΔTGPI1 (Fig. 3B), and restoration in both ΔTGPI1 plus PI1α-GST and ΔTGPI1 plus PI1β-GST transgenics (Fig. 3C and D). TGPI1 is secreted via dense granules into the parasitophorous vacuole (45) (Fig. 3A), and expression of TGPI1α or TGPI1β in ΔTGPI1 knockout mutants demonstrated that both isoforms were also secreted into the parasitophorous vacuole (Fig. 3C and D). Interestingly, TGPI1 was also observed in the cytoplasm of a subset (57% ± 5% in three independent replicates) of infected host cells, along with another dense-granule protein, GRA1 (Fig. 3E). In contrast, a P30-OVA transgene (43) expressed in the same cells was not seen in the host cell cytoplasm despite being abundantly present in the
parasitophorous vacuole (Fig. 3F). P30-OVA, which consists of the P30 (SAG1) signal sequence fused to the model antigen ovalbumin, serves as a default secretory marker lacking specific targeting signals. Moreover, TgPI1 localization to the host cytoplasm was observed in a majority of infected host cells, including those containing small, as well as large, parasitophorous vacuoles, although fluorescence is usually stronger in the latter, presumably due to the presence of more parasites secreting TgPI1. The fact that TgPI1 and GRA1 were seen in the cytoplasm of host cells containing a range of parasitophorous vacuoles suggests that cytoplasmic transfer was not a result of vacuolar permeabilization resulting from imminent parasite egress. For further validation, we transfected ΔTgPI1 parasites with plasmids encoding TgPI1α fused to monomeric red fluorescent protein (mRFP) or TgPI1β fused to yellow fluorescent protein (YFP). Both of these TgPI1–fluorescent-protein fusions were observed in the cytoplasm of live infected HFF (Fig. 3G and H), ruling out the possibility that transfer of TgPI1 to the host cytoplasm occurred during fixation. These findings suggest that TgPI1 has access to potential targets in the host cytoplasm, thus opening the possibility that TgPI1 suppresses host proteases during intracellular growth.

ΔTgPI1 tachyzoites exhibit normal replication but enhanced differentiation in vitro. To determine if TgPI1 influences parasite replication, confluent HFF monolayers were inoculated with an equivalent number of wild-type RH strain tachyzoites, RHΔHXGPRT parasites, ΔTgPI1 knockouts, or clonal parasite lines complemented with TgPI1α-GST or TgPI1β-GST. ΔHXGPRT parasites display a slight growth defect (Fig. 4A) (a doubling time of 9.2 ± 0.9 h for RHΔHXGPRT versus 7.1 ± 0.6 h for wild-type RH), as previously described (9). However, ΔTgPI1 knockout parasites, which express HXGPRT, displayed a doubling time (~7.8 ± 0.4 h) that was indistinguishable from that of wild-type RH. Strains expressing TgPI1α and TgPI1β also exhibited doubling times similar to that of the wild type (~6.7 and 7.7 h, respectively). Thus, TgPI1 expression appears to have no significant impact on T. gondii tachyzoite replication in vitro. Since RHΔHXGPRT has different growth properties due to the lack of HXGPRT expression, the wild-type RH strain is used here as the reference strain.

T. gondii strain RH does not form mature bradyzoite cysts in mice but can be induced to differentiate in vitro under stress conditions, such as exposure to alkaline pH (4, 5, 17, 18, 52, 54, 57). Bradyzoite-specific antigens are detected as early as 12 to 24 h postinduction (P. H. Davis, unpublished data), and cyst wall development can be visualized by staining with D. biflorus lectin (6). To test if TgPI1 plays a role in stage differentiation, confluent monolayers of HFF were infected with wild-type RH, the ΔHXGPRT or ΔTgPI1 knockout mutant, and complemented parasite lines and assayed for differentiation after 48 h of incubation at pH 8.1 (Fig. 4B). ΔTgPI1 knockout parasites showed a 64-fold upregulation of the bradyzoite-specific enolase isozyme ENO1 (Fig. 4B) (27) relative to wild-type T. gondii. Knockout parasites also showed a similar hyperinduction of other bradyzoite stage-specific markers, including P18/SAG4.2, BAG1, and LDH2 (upregulated 13-, 25-, and 30-fold, respectively).

Low levels of Dolichos staining were observed in alkali-treated T. gondii strain RH cultures (Fig. 4C, left), perhaps associated with the intravacuolar membrane network (34), but only ~8% of these vacuoles showed evidence of bradyzoite cyst differentiation, including (i) high levels of Dolichos lectin staining on the vacuolar surface, (ii) reduced replication (2 to 8 parasites within the vacuole versus 16 to 32 in tachyzoite-containing vacuoles), (iii) rounded parasite morphology lacking the “rosette” organization typical of tachyzoites, and (iv) rounded, cyst-like vacuoles
(Fig. 4C). In contrast, 74% of vacuoles containing ΔTgPI1 knockouts were strongly Dolichos
positive, and complementation with either TgPI1α or TgPI1β substantially reversed the
differentiation phenotype (Fig. 4B). Thus, deletion of TgPI1 enhances bradyzoite differentiation
of RH strain parasites in vitro. While other T. gondii mutants are also prone to enhanced
bradyzoite differentiation (cf. the ΔHXGPRT mutant in Fig. 4B), the ΔTgPI1 effect is
particularly profound. It is possible that TgPI1 inhibits proteases involved in bradyzoite cyst
formation, as the cyst wall contains chitin (6) and there is ample precedent for proteolytic
regulation of chitin in other systems (7, 13, 37).

Deletion of TgPI1 increases the parasite tissue burden in infected mice. To assess the importance of
tgPI1 in vivo, BALB/c mice were inoculated with freshly harvested tachyzoites by intraperitoneal
injection, and the liver, spleen, and brain were harvested from euthanized animals during the acute phase
of infection 7 days postinoculation. The parasite tissue burden was measured by quantitative PCR.
Infection with RH strain parasites develops rapidly and is typically fatal on approximately day 8
postinoculation, which is prior to the initiation of the chronic phase of infection (days 10 to 12 for
cystogenic strains [15]). As shown in Fig. 4D, ΔTgPI1 tachyzoites reproducibly displayed significantly
higher parasite burdens than the RH strain in all tissues (three independent experiments).
Complementation with either TgPI1α or TgPI1β reduced the parasite burden to the level observed in wild-
type controls in the spleen. A similar trend was seen in the liver, although ΔTgPI1β did not fully reverse
the elevated parasite burden phenotype. Neither TgPI1α nor TgPI1β restored the normal parasite burden
in the brain, which is low and highly variable, thus possibly explaining the apparent lack of
complementation. Alternatively, it is possible that TgPI1α and TgPI1β each target different proteases in
the brain, and therefore, complementation with both isoforms would be necessary to restore normal
infection levels.

Most other dense-granule protein mutants that have been characterized to date exhibit either no phenotype
(GRA5) (27) or reduced virulence (GRA2, GRA3, and GRA6) (12, 35, 36). In contrast, deletion of TgPI1
appears to enhance the parasite burden during acute tachyzoite infection of mice. This phenotype is
reminiscent of a recently reported type II strain GRA15 knockout mutant, which replicates to greater
levels in mice than wild-type parasites (48). GRA15 is associated with the PV and appears to have access
to the cytoplasm of infected host cells, where it activates nuclear translocation of NF-κB in a parasite
strain-dependent manner, resulting in upregulation of several genes, including the proinflammatory
cytokine interleukin 12 (IL-12). Rapid production of IL-12 during early infection with type II parasites
induces gamma interferon (IFN-γ), which suppresses parasite growth (38). Conversely, deletion of
GRA15 delays production of IL-12 and IFN-γ, thus permitting rapid growth and a higher parasite burden
in vivo. While TgPI1 ablation also leads to a higher parasite burden in vivo, precisely how TgPI1 affects
parasite growth in mice remains to be determined. Although higher levels of TgPI1 expression, especially
TgPI1α, in the Pru strain correlates with the slower growth and reduced virulence of this type II strain,
strain-specific virulence genes have not been mapped to chromosome Ib, where TgPI1 is encoded. Future
studies focused on the identification of proteases targeted by TgPI1 during infection should provide
further insight into how TgPI1 influences T. gondii differentiation and growth in vivo.
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References


Figures

**Fig 1**

TgPI1α and TgPI1β are mRNA splice variants. (A) Western blotting with anti-TgPI1 revealed two isoforms (TgPI1α and TgPI1β) in T. gondii strains RH (type I), Pru (type II), and VEG (type III). Each lane contained 10^6 parasite equivalents. (B) Pulse-chase labeling of intracellular RH strain tachyzoites with ^35^S-labeled amino acids showed no evidence of posttranslational modification. (C) MALDI-TOF analysis of the two intact isoforms revealed an exact molecular mass difference of 2,775 Da (the average of three independent replicates). (D) MALDI-TOF analysis of tryptic fragments revealed a peak at m/z 1,652.7 specific to TgPI1α (arrow). (E) RT-PCR showed reverse transcriptase (RT)-dependent amplification of 887- and 815-nt bands in samples containing total tachyzoite RNA (lane 1) or a cDNA library (lane 4); amplification from genomic DNA yielded a 2,973-nt band (lane 5). (F) Schematic representation of the two alternatively spliced variants of TgPI1. The boxes represent exons (open, untranslated; filled, coding; gray, alternatively spliced exon 6). The white stars indicate predicted Kazal domains.
Fig 2

Targeted deletion of the *TgPI1* locus. (A) (Top) Scale diagram of the 12.3-kb EcoR I fragment spanning the *TgPI1* genomic locus. The boxes represent all nine *TgPI1* exons (open, untranslated; filled, coding; gray, alternatively spliced exon in *TgPI1α*). (Bottom) *dhfrHXGPRTdhfr* targeting construct used for gene disruption. The shaded parallelograms denote identity to sequences flanking *TgPI1* (2.5 kb upstream and 3.6 kb downstream). Arrowheads, primers for PCR screening; bars, probes for Southern hybridization. (B) PCR of the *TgPI1* locus in RHΔHXGPRT parental parasites and one (of several) Δ*TgPI1* mutant clones. The *TgPI1* locus is present in parental parasites, but not the mutant (P1-P2) or the targeting construct integrated at the genomic locus (P3-P5 and P3-P4). Panel A shows the primer positions. (C) Southern blot illustrating deletion of the genomic *TgPI1* locus in two Δ*TgPI1* clones and integration of *TgPI1α* or *TgPI1β* transgenes at heterologous sites in complemented lines (panel A shows the probes). (D) Western blot with rabbit anti-*TgPI1* illustrating expression of both *TgPI1α* and *TgPI1β* isoforms in parental parasites, loss in Δ*TgPI1* mutants, and expression of *TgPI1α*-GST or *TgPI1β*-GST fusion proteins in parasites expressing complementation constructs. (E) Microarray analysis of steady-state transcript abundance (log2 values) for *TgPI1* and other protease inhibitor genes in parental RHΔHXGPRT and Δ*TgPI1* parasites. The gray shading indicates background expression levels.
Fig 3

*TgPII1α* and *TgPII1β* are secreted into the parasitophorous vacuole and the host cell cytoplasm. (A to D) Immunofluorescence with anti-*TgPII1* demonstrates staining of the parasitophorous vacuole in parental parasites, no staining in Δ*TgPII1* mutants, and restoration in knockouts complemented with either *TgPII1α*-GST or *TgPII1β*-GST. The arrows indicate vacuoles containing parasites expressing *TgPII1*. (E and F) Colocalization of *TgPII1* and GRA1, but not P30-OVA, in the cytoplasm of host cells infected with parasites expressing a P30-OVA transgene. (G and H) Identification of *TgPII1α* and *TgPII1β* in the host cell cytoplasm using fluorescent protein reporters (in live cell cultures). The closed arrowheads indicate staining of the parasitophorous vacuole and cytoplasm of host cells. The open arrowheads show vacuoles with no evidence of *TgPII1* staining in the cytoplasm of host cells.
Phenotypic effects of ΔTgPI1 in vitro and in vivo. (A) ΔTgPI1 mutants are identical to wild-type parasites with respect to proliferation in vitro, as assessed by scoring parasite replication at various times postinfection (the average log₂[parasite number] for 100 intracellular vacuoles, in triplicate). Doubling times were ~6.7 to 7.8 h for wild-type RH, ΔTgPI1 knockout mutants, and ΔTgPI1 plus PI1α- and ΔTgPI1 plus TgPI1β-complemented lines, in contrast to the slightly crippled replication rate of RHΔHXGPRT (9.2 ± 0.9 h). The error bars indicate standard deviations (SD). (B) Enhanced in vitro differentiation of ΔTgPI1. Treatment for 48 h at pH 8.1 induced expression of the bradyzoite differentiation marker ENO1 (measured by quantitative PCR) and surface staining with D. biflorus lectin. Differentiation of ΔTgPI1 was more extensive than that of ΔHXGPRT and much more than was observed in wild-type parasites. Partial complementation was observed in both ΔTgPI1 plus PI1α and ΔTgPI1 plus PI1β; ND, not determined. Ave., average; SE, standard error. (C) Morphology of alkaline-treated parasite cultures stained with fluorescent Dolichos lectin. Weak intravacuolar staining was observed for ~70% of wild-type parasites (left), while ~8% displayed strong Dolichos staining of the vacuolar surface and rounded bradyzoite cyst-like morphology (the remaining vacuoles were unstained). Approximately 70% of ΔTgPI1 vacuoles displayed bradyzoite morphology, even when extensive replication was observed (far right). (D) Enhanced virulence of ΔTgPI1 in vivo. Female BALB/c mice inoculated with 10^3 tachyzoites showed higher tissue burdens of ΔTgPI1.
than of the wild-type in the liver, spleen, and brain at day 7 postinfection. Full or partial complementation was observed for both $\Delta TgPI1$ plus $PI1\alpha$ and $\Delta TgPI1$ plus $TgPI1\beta$ in the liver and spleen, but not the brain (the bars show means ± SD for 2 or 3 experiments involving 2 to 5 mice per sample). The asterisks indicate a $P$ value of ≤0.05 relative to RH (wild type). See the text for further discussion.