We have characterized a Kazal family serine protease inhibitor, *Toxoplasma gondii* protease inhibitor 1 (TgPI-1), in the obligate intracellular parasite *Toxoplasma gondii*. TgPI-1 contains four inhibitor domains predicted to inhibit trypsin, chymotrypsin, and elastase. Antibodies against recombinant TgPI-1 detect two polypeptides, of 43 and 41 kDa, designated TgPI-1^4^3 and TgPI-1^4^, in tachyzoites, bradyzoites, and sporozoites. TgPI-1^4^ and TgPI-1^4^ are secreted constitutively from dense granules into the excreted/secreted antigen fraction as well as the parasitophorous vacuole that *T. gondii* occupies during intracellular replication. Reombinant TgPI-1 inhibits trypsin, chymotrypsin, pancreatic elastase, and neutrophil elastase. Immunoprecipitation studies with anti-rTgPI-1 antibodies reveal that recombinant TgPI-1 forms a complex with trypsin that is dependent on interactions with the active site of the protease. TgPI-1 is the first anti-trypsin/chymotrypsin inhibitor to be identified in bradyzoites and sporozoites, stages of the parasite that would be exposed to proteolytic enzymes in the digestive tract of the host.

*Toxoplasma gondii* commonly infects humans and occasionally causes opportunistic disease. Recrudescence of a latent infection in immunodeficient individuals can result in encephalitis (1). Transplacental transmission of *T. gondii* can cause spontaneous abortion, mental retardation, and blindness (2).

*T. gondii* is primarily acquired through the ingestion of sporulated oocysts, containing sporozoites, shed by the definitive host (felids) or by ingestion of undercooked meat harboring bradyzoite tissue cysts. The cyst wall is digested during transit through the gastrointestinal tract, releasing the bradyzoites/sporozoites, which penetrate the intestinal epithelium where they immediately differentiate into rapidly dividing tachyzoites. Tachyzoites disseminate and proliferate during the acute stage of infection before differentiating into bradyzoites, which encyst in muscle tissue and the central nervous system thereby establishing a chronic infection (3).

Because *T. gondii* transits through the digestive tract and is resistant to physiological levels of trypsin (4), it has been speculated that *T. gondii* secretes protease inhibitors that aid in protecting the parasite from the proteolytic enzymes, trypsin and chymotrypsin, found within the lower intestine. Previously, TgPI^4^ (5) was identified in *T. gondii*. We have independently identified the same protease inhibitor, termed TgPI-1. Until now, expression of protease inhibitor polypeptides has not been demonstrated in bradyzoites or sporozoites, stages of the parasite most likely to be exposed to the proteolytic environment of the digestive tract.

As obligate intracellular parasites, host cell invasion and subsequent proliferation are essential to the lifecycle of *T. gondii*. During the lytic cycle of replication parasite attachment to the host cell is accompanied by the discharge of micronemes, small cigar-shaped vesicles located at the apical end of the parasite. Next, rhoptry (ROP) proteins are discharged and serve to aid in the establishment of the parasitophorous vacuole (PV), a protected compartment within the host cell in which the parasites reside. Once inside the host cell, the parasite releases proteins from dense granules (DGs) into the PV. Although ten DG proteins have been identified (6, 7), the precise functions of DG proteins remain poorly understood.

N-terminal sequencing of proteins found in excreted/secreted antigen (ESA) fractions reveal the presence of TgPI-1, a protein belonging to the Kazal family of serine protease inhibitors. This family of protease inhibitors is defined by a set of conserved disulfide bonds that form a rigid structure exposing the reactive site P1 residue for interaction with its target protease (8–10). TgPI-1 contains four inhibitor domains, which are predicted to inhibit chymotrypsin, trypsin, and elastase.

Serine protease inhibitors are classified according to structure and mechanism of inhibition (8–10). The SERPIN, Kunitz, and Kazal families represent some of the most widely studied families of serine protease inhibitors, some of which function in viral or parasite pathogenesis. For example, several SERPINS identified from poxvirus have been shown to inhibit inflammation and apoptosis and function in determination of viral host range (11). A Kunitz family serine protease inhibitor isolated from the hookworm *Ancylostoma ceylanicum* has activity against chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin and may contribute to the ability of the parasite to evade the immune system and provide protection during its residence within the small intestine (12). Kazal inhibitors have

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1 The abbreviations used are: TgPI, *T. gondii* protease inhibitor; rTgPI-1, recombinant TgPI-1; ROP, rhoptry; PV, parasitophorous vacuole; DG, dense granules; ESA, excreted/secreted antigen; pNA, *p*-nitroaniline; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BAPTA-AM, bis(o-aminoethoxy)ethane-N,N,N’,N’-tetra-acetic acid sodium salt; HFF, human foreskin fibroblasts; PBS, phosphate-buffered saline; mAb, monoclonal antibody; IPTG, isopropyl-β-D-galactopyranoside; EST, expressed sequence tag; RorTgPI-1, rabbit antibody generated against rTgPI-1.
been found in mammals (pancreatic secretory trypsin inhibitors, mammalian seminal acrosin inhibitors) (13), birds (avian ovomucoids, chicken ovoinhibitor) (8), leeches (leech-derived trypsin inhibitor) (14), and insects (rhodniin) (15) and used extensively in vitro to study the interactions of aminopeptidases with their substrates. However, little work has been published on the function of Kazal inhibitors in vivo.

Here we show that TgPI-1 protein is expressed in *T. gondii* tachyzoites, bradyzoites, and sporozoites and is secreted from *T. gondii* parasites. Western blotting (Figure 1) was used to confirm the presence of TgPI-1 in total parasite protein extracts. Extracted peptides were dried down and redissolved in 2 μl of 50% acetonitrile and 0.3% trifluoroacetic acid, mixed with matrix (α-cyano-4-hydroxycinnamic acid), and deposited on the mass spectrometry (MS) target plate according to a general two-layer method (22). MS was performed on a matrix-assisted laser desorption/sonication time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems Voyager DE-STR). The spectra of peptide mass fingerprints were acquired in the positive reflection mode with delayed extraction. The spectra were calibrated by external standard sample (ProteoMass peptide calibration kit) deposited on the MS plate adjacent to the Tg-PI-1 spot.

Peptide mass fingerprinting data from MALDI-TOF were used to search against NCBI data base via the MS-Fit algorithm (prospector.ucsf.edu). The search resulted in a match to TgPI-1 (nine matching peptides at mass tolerance of 50 ppm, Mowse score 9.15 × 102, 22% sequence coverage).

**Secretion Assays**—Secretion analyses were performed essentially as described previously (23). Briefly, 3 × 106 tachyzoites were resuspended in invasion media (DMEM, 1.5 g/liter sodium bicarbonate, 20 mM HEPES, pH 7.4, and 3% fetal bovine serum) and incubated for 2 min at 37 °C with 100 μM BAPTA-AM or invasion media alone before being transferred to an ice water bath. Parasites were removed by centrifugation (twice at 10,000 × g for 3 min at 4 °C), and culture supernatants were concentrated 20-fold in C-10 concentrators, aliquoted, and stored at −80 °C until use.

**Immunolocalization of TgPI-1**—Immunofluorescence of intracellular parasites was performed essentially as described previously (24). Briefly, intracellular parasites were allowed to invade monolayers of HFF for various time intervals, fixed in 4% formaldehyde, 0.027% glutaraldehyde, PBS for 20 min at 25 °C, and fully permeabilized with 0.1% Triton X-100. PBS. TgPI-1 was detected using rabbit polyclonal sera (RarTgPI-1) diluted 1:500, GRA4 and GRA1 were detected with 1:500 dilution of anti-granzyme A (clone 106). Antibody staining was done exactly as described for intracellular parasites.

Vaccines Fractionations—Membranous fractions were isolated from vacuoles as described previously (25). Briefly, tachyzoites were inoculated onto HFF monolayers and incubated 16 h. Monolayers were washed twice with PBS to remove extracellular tachyzoites, and vacuoles were lysed by passage through 23- and 25-gauge syringes. Liberated vacuoles were removed by centrifugation at 1,000 × *g* for 15 min at 25 °C. The low speed supernatant was centrifuged at 10,000 × *g* for 2 h at 4 °C to separate membranous fractions (pellet) and soluble components (supernatant). Equivalent amounts of pellet and supernatant were analyzed by Western blotting.

**Expression and Purification of TgPI-1**—The following primers were used to amplify TgPI-1 from a cDNA library: 5′-GACTGGATCCCGTCCGCCCCGAAAAGCGAGG-3′ and 5′-GTTGATGCGGCGGCTCGAG-3′. PCR products were gel-purified using Qiagen spin columns, ligated into pQE30 vector digested with *KpnI-BamHI*, and transformed into *E. coli* JM109. Positive transformants were confirmed by sequencing both strands of the insert.

Bacteria harboring TgPI-1 in pQE30 were induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 30 min. 4 °C, washed twice with 1 ml of lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole), and sonicated 10 min. 4 °C, and the resulting supernatant was loaded onto a 1-mL nickel-nitrioltriacetic acid column and eluted into four 0.5-mL fractions with elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM imidazole). For activity assays, *rTgPI-1* was dialyzed against PBS to exchange buffers.
polyethylene glycol 4000. 0.4 mM Suc-Ala-Ala-Pro-Lys-pNA, pancreatic elastase assay buffer: 0.1 mM Tris-Cl, pH 8.0, 0.5 mM Suc-Ala-Ala-Pro-Phe-pNA, human neutrophil elastase buffer: 0.2 mM Tris-Cl, pH 8.0, 0.2 mM Suc-Ala-Ala-Pro-Ala-pNA. Experiments were performed at least twice, in triplicate, and reaction velocities were linear over the course of the reaction. Initial velocities were measured by monitoring absorbance at 405 nm on a Vmax™ Molecular Devices kinetic microplate reader. Kinetics were determined via monitoring absorbance at 405 nm. Double-reciprocal Lineweaver-Burke plots of 1/V versus 1/[S] were used to determine K_m of each substrate for its partner protease.

Immunoprecipitations—Protease-inhibitor complexes were prepared by incubation of purified trypsin (400 ng) with ~1.5-fold molar excess of rTgPI-1 (400 ng) in PBS for 30 min at 25 °C in a final volume of 10 µl. Complexes were immunoprecipitated by addition of 200 µl of radiolabeled precipitate assay buffer (1 mM Tris, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 5 mM EDTA) and incubation with 2.5 µl of mouse anti-rTgPI-1 antisera for 16 h at 4 °C with gentle agitation. Protein G-Sepharose beads (2.5% final v/v) were added and incubated for 2 h at 25 °C. Beads were washed four times with 1 ml of radiolabeled precipitate assay buffer, aspirated, and resuspended in 100 µl of 0.2% BSA, 10% glycerol, 32 mM Tris, pH 6.8, 0.02 mg/ml bromophenol blue, 2% 2-mercaptoethanol.

SDS-PAGE and Western Blotting—SDS-PAGE was performed on 12.5% minigels (Bio-Rad) under reducing conditions, and proteins were transferred to nitrocellulose membranes for 40 min at 10 V using a Transblot SD semi-dry transfer cell (Bio-Rad). Western blots were developed by chemiluminescence as described previously (26).

RESULTS

TgPI-1 belongs to the Kazal Family of Protease Inhibitors—Previously, putative secretory proteins were identified utilizing sub-cellular fractionation techniques and isolation of excreted/secreted antigens (ESA) in parasite culture supernatants (27). N-terminal sequencing of putative secretory proteins revealed the presence of T. gondii protease inhibitor 1 (TgPI-1) in ESA. BLAST analysis of the T. gondii expressed sequence tag (EST) database with the TgPI-1 nucleotide sequence reveals that the TgPI-1 sequence was present in the database with 13 tachyzoite ESTs and no bradyzoite ESTs.

The TgPI-1 gene is predicted to encode a 30-kDa protein containing four protease inhibitor domains and an N-terminal signal sequence (Fig. 1A). TgPI-1 is predicted to be a serine protease inhibitor belonging to the Kazal family whose members characteristically have multiple protease inhibitor domains (8–10). This family of protease inhibitors is defined by a set of conserved disulfide bonds that form a rigid structure exposing the reactive site P1 residue for interaction with its cognate protease domain. Disulfide linkages are indicated by solid lines above the sequence alignments.

mRNA for TgPI-1 is present in both tachyzoites and bradyzoites. Antibodies were raised against recombinant TgPI-1 (rTgPI-1). Rabbit antibodies generated against rTgPI-1 (RatTgPI-1) recognize two polypeptides in extracellular tachyzoites, bradyzoites, and sporozoites, TgPI-143 and TgPI-141, named according to their apparent molecular weight as determined by SDS-PAGE (Fig. 2B, lanes 1–5).

TgPI-143 and TgPI-141 Secretion into ESA Is Unaffected by BAPTA-AM—TgPI-1 possesses a hydrophobic N-terminal sequence that has the properties of a signal sequence, suggesting that it might be a secretory protein. In an effort to determine whether TgPI-1 was secreted, two-dimensional gels of T. gondiiESA fractions consist predominantly of secreted micronemal and EG proteins. Calcium chelators such as BAPTA-AM specifically block microneme secretion without markedly affecting EG secretion (16). To determine if TgPI-143 and TgPI-141 are of micronemal or EG origin, ESA fractions obtained from untreated and BAPTA-AM treated parasites were resolved by SDS-PAGE and probed with RatTgPI-1. As a control, release of the micronemal protein, TgMIC2 (115 kDa), which is proteo-

FIG. 1. TgPI-1 belongs to the Kazal family of serine protease inhibitors. A, TgPI-1 contains a signal sequence and four inhibitor domains. TgPI-1 has an N-terminal signal sequence (SP) and four inhibitor domains that have been modeled according to the three-dimensional structure of leech-derived trypsin inhibitor using Swiss-Model software. This model is intended to schematically depict the general structure of the inhibitor domains and has not been rigorously optimized. Each domain is defined by three disulfide bonds, generating a rigid structure in which the P1 residue (indicated by the single-letter amino acid code) is left exposed to interact with its cognate protease (indicated under each domain). B, sequence alignment of Kazal family inhibitor domains. TgPI-1a-d: protease inhibitor domains of TgPI-1 (accession number AF121778), NcPI: Neospora caninum protease inhibitor (V. B. Carruthers, unpublished), Rh1d and Rh1d2: the first and second protease inhibitor domains of rhodinia (accession number Q06684). Ldlti: leech-derived trypsin inhibitor (accession number P68042). Bdh3: Bdelin B-3 (accession number P09865). Kazal family proteases are defined by conserved cysteine residues (underscored). Reactive P1 residues (in boldface) dictate the target protease. Disulfide linkages are indicated by solid lines above the majority sequences.
nitrocellulose, and probed with R

T. gondii Discharge

After invasion of the host cell, DG proteins

been speculated that DG proteins play a role in the maintenance and remodeling of the PV as well as nutrient acquisition (28). Dual label immunofluorescence of timed invasions was used to assess the kinetics of TgPI-1 secretion into the PV.

Freshly isolated tachyzoites were inoculated onto a monolayer of human foreskin fibroblasts (HFF) for 1 min and incubated for varying intervals of time before they were fixed, permeabilized, and probed with RorTgPI-1 and a mouse mAb against GRA1 (mAbTg17–43) or GRA4 (WU# 950). Ten minutes post-invasion, RorTgPI-1 and mAb Tg17–43 exhibited intense staining on either side of the apical end of the parasites (Fig. 4B) in 45% (45/101) of the PVs observed. At 60 min post-invasion, TgPI-1 and GRA1 are completely filled the PV (Fig. 4D) in virtually all PVs (not quantified). Similar patterns were also observed in single-labeling experiments using RorTgPI-1, mAb Tg17–43, or WU# 950 (data not shown). These results indicate that the kinetics of TgPI-1 secretion into the PV follows the timing of DG discharge.

TgPI-1 Remains Soluble after Secretion into the PV—After secretion into the PV, DG proteins can be targeted to the PV membrane (29, 30) or the intravacuolar network of tubular membranes within the PV (25) or remain soluble (31). The solubility characteristics of a DG protein are important for predicting whether the protein will remain confined to the host cell after parasite egress or whether it will be released to

Fig. 2. TgPI-1 is expressed in multiple life stages of T. gondii. A, TgPI-1 mRNA is present in tachyzoites and bradyzoites. Bradyzoite and tachyzoite mRNA were used in RT-PCR with primers specific for T. gondii superoxide dismutase (TgSOD, lanes 1 and 2) or TgPI-1 (lanes 3 and 4). B, TgPI-1-43 and TgPI-1-41 are detected by Western blotting in tachyzoites and bradyzoites. Tachyzoite (Tz) lysate (5 × 10⁶/lane 1, 3 × 10⁵/lane 2, 1 × 10⁵/lane 3), bradyzoite lysate (1000 cysts, ~1 × 10⁶ bradyzoites (Bz), lane 4), and fully sporulated oocysts (5 × 10⁶ sporozoites (Sz), lane 5) were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose, and probed with RorTgPI-1 (lanes 1 and 5).

Fig. 3. TgPI-1 secretion into ESA is calcium independent. A, Western blot of two-dimensional gel electrophoresis of ESA. ESA fractions were separated in the first dimension using PROTEAN isoelectric focusing system employing pH gradient strips (pH 4–7) and then resolved in the second dimension on a 10–20% linear gradient SDS-PAGE gel, transferred to nitrocellulose, and probed with rabbit TgPI-1 antibodies. An asterisk marks residual signal from a previous blot. B, colloidal Coomassie stain of two-dimensional gel electrophoresis of ESA. A sister two-dimensional gel of ESA was run as described in A and stained with colloidal Coomassie. The circled band is TgPI-1. C, secretion of the microneme protein, MIC2, is blocked by BAPTA-AM. Tachyzoite (Tz) lysate (10⁵, lane 1), 5 ng of recombinant TgPI-1 (rTgPI-1), ESA (E, lane 3), and ESA from BAPTA-AM-treated parasites (E/B, lane 4) were resolved by 12.5% SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with rabbit anti-MIC2 antibodies. D, secretion of the DG protein, GRA1, is unaffected by BAPTA-AM. The membrane from C was stripped and probed with anti-GRA1 antibodies, mAb Tg17–43. E, secretion of TgPI-1-43 and TgPI-1-41 are unaffected by the calcium agonist BAPTA-AM. The membrane from C was stripped and probed with RorTgPI-1.
potentially act in the extracellular environment of the tissues surrounding the site of infection. Differential centrifugation was used to determine the intravacuolar localization of TgPI-1 and TgPI-1.

HFF monolayers were infected with tachyzoites for 24 h and washed to remove extracellular parasites. Vacuoles containing 8–16 parasites were mechanically lysed and centrifuged to remove the intact parasites. The low speed supernatant was centrifuged at 100,000 × g to pellet membranes containing PV membrane and tubular membrane networks. The soluble and membrane fractions were analyzed by Western blotting. As controls, GRA1, a soluble vacuole component, and GRA2, which distributes evenly between the membrane and soluble fractions (Fig. 5A, lanes 2 and 3), and GRA2, which distributes evenly between the membrane and soluble fractions (Fig. 5B, lanes 2 and 3). TgPI-1 and TgPI-1 were detected exclusively within the soluble fraction (Fig. 5C, lanes 2 and 3). These results indicate that TgPI-1 and TgPI-1 remain soluble after secretion into the PV. In addition, TgPI-1 and TgPI-1 do not appear to be proteolytically processed or otherwise modified upon their release into the PV.

Recombinant TgPI-1 Is a Broad-spectrum Serine Protease Inhibitor—Based on homology with other Kazal family members, TgPI-1 is predicted to contain four serine protease inhibitor domains (Fig. 1A). In the Kazal family, the cognate protease is dictated by the amino acid occupying the P1 position. TgPI-1 has two domains where arginine residues, predicted to inhibit trypsin, occupy the P1 position and two additional domains containing leucine residues at the P1 position. Leucine at P1 has been observed to inhibit chymotrypsin and elastase (reviewed in Refs. 8, 10, and 33).

Recombinant TgPI-1 (rTgPI-1) was expressed, purified from Escherichia coli, and tested for inhibitory activity against trypsin, chymotrypsin, and elastase. Proteases were incubated with increasing amounts of inhibitor, and Ki values were determined by monitoring cleavage of the peptide substrate. As predicted on the basis of the P1 position of each domain, rTgPI-1 inhibited chymotrypsin, trypsin, pancreatic elastase, and neutrophil elastase. The strongest inhibitory activity was observed against trypsin and chymotrypsin with Ki values of 0.035 and 0.35 nM, respectively (Table I). Recombinant TgPI-1 (rTgPI-1) also inhibits pancreatic elastase as well as human neutrophil elastase. Although the Ki values for pancreatic elastase (Ki = 15 nM) and human neutrophil elastase (Ki = 49 nM) were markedly higher than the Ki values observed for both trypsin and chymotrypsin (Table I), both proteases were significantly inhibited by rTgPI-1. Pancreatic elastase was inhibited 80% when preincubated with a 2-fold molar excess of inhibitor, whereas neutrophil elastase was inhibited 50% in the presence of a 2-fold molar excess and almost 80% when 4-fold
molar excess of inhibitor was added (data not shown).

It is possible that the inhibition observed was actually due to degradation of the test protease by a contaminating E. coli protease that co-purified with our inhibitors. To rule out this possibility, proteases were incubated with the inhibitors under the same conditions used to assess inhibition. Protease/inhibitor mixes were then separated on SDS-PAGE gels and stained to confirm that the test protease was intact. In all cases, the protease amounts were unchanged after incubation with the inhibitors (data not shown).

Recombinant TgPI-1 Forms a Stable Complex with Trypsin through Active Site Interactions—The closely related Kazal and Kunitz inhibitors act as pseudosubstrates consisting of an exposed reactive loop, which fits in the active site cleft of the target enzyme (33). In most cases binding is rapid and tight. If TgPI-1 inhibits trypsin by the mechanism described above, it should be possible to isolate trypsin-rTgPI-1 complexes. Additionally, modification of the trypsin active site should inhibit complex formation. Trypsin-rTgPI-1 complexes were isolated by incubation of inhibitor with enzyme followed by immunoprecipitation with mouse anti-rTgPI-1 antibodies. Immunoprecipitated material was then analyzed by Western blotting with rabbit anti-trypsin antibodies. As expected for complex formation, purified trypsin co-immunoprecipitated with rTgPI-1 and mouse anti-rTgPI-1 antibodies (Fig. 6A, lane 3). Trypsin was not detected when mouse anti-rTgPI-1 antibodies were used in immunoprecipitations in the absence of rTgPI-1 (Fig. 6A, lane 2). Preincubation of trypsin with (p-4-amidinophenyl)methylsulfonyl fluoride, a compound that covalently modifies the trypsin active site (34), blocked complex formation (Fig. 6B, lane 4). Furthermore, preincubation of trypsin with soybean trypsin inhibitor, an inhibitor known to interact with the active site of trypsin (35, 36), blocked complex formation between rTgPI-1 and trypsin (Fig. 6B, lane 3). Preincubation of trypsin with buffers alone did not interfere with the ability of rTgPI-1 to form a complex with trypsin (Fig. 6B, lanes 1 and 2). These experiments indicate that rTgPI-1 forms a complex with purified trypsin through active site interactions.

**DISCUSSION**

Serine proteases and their cognate inhibitors play fundamental roles in cellular biology with functions ranging from digestion, apoptosis, proteolytic processing, wound healing, and cellular and extracellular remodeling (8–10, 33). Serine protease inhibitors can be grouped into several families, including the SERPIN, Kazal, and Kunitz families of inhibitors, based on their structural homologies and mechanism of inhibition. The SERPINS have been most extensively studied, whereas little work has been published on the cellular biology of the Kazal family of inhibitors.

Kazal inhibitors have been identified in a wide variety of organisms, including birds, mammals, insects, and leeches (8, 13–15). Because Kazal inhibitors act as pseudosubstrates, they have been used extensively to elucidate interactions between serine proteases and their substrates (8–10, 33); however, little work has centered on the in vivo function of this family of inhibitors. We have identified a Kazal inhibitor, TgPI-1, in *T. gondii*, a parasite amenable to genetic manipulation and easily cultured in vitro under a wide variety of conditions.

Western blots indicate that two polypeptides, named TgPI-143 and TgPI-141, according to their apparent molecular weight, are detected in Toxoplasma cell lysates by antibodies raised against recombinant TgPI-1. Currently, the relationship between TgPI-143 and TgPI-141 is unknown. TgPI-143 may represent the full-length polypeptide that is proteolytically processed to the smaller form, TgPI-141. Alternatively, it is possible that the full-length TgPI-143 is glycosylated to TgPI-143. The ratio between TgPI-143 and TgPI-141 can vary between lysate preparations, although it is unclear why this is the case. The presence or absence of protease inhibitors in the parasite purification protocol does not affect the ratio of TgPI-143 to TgPI-141 (data not shown), suggesting that the two species are not generated during sample preparation.

TgPI-143 and TgPI-141 are secreted into ESA fractions by extracellular tachyzoites in a BAPTA-AM-insensitive manner, a characteristic of DG proteins. Additionally, indirect immunofluorescence of *T. gondii* as it invades host cells reveals that TgPI-1 is secreted into the parasitophorous vacuole by intracellular parasites. It is likely that TgPI-1 is also secreted by...
bradyzoites and sporozoites in a manner similar to that observed in tachyzoites. Dense granule proteins are constitutively secreted by extracellular tachyzoites, and secretion into the PV is slightly up-regulated upon invasion (24). In vitro studies indicate that rTgPI-1 is active against trypsin, chymotrypsin, pancreatic elastase, and neutrophil elastase. Knowledge regarding the expression and secretion of TgPI-1 allows us to predict what types of host cell tissues and proteases this broad-spectrum inhibitor is likely to encounter.

_T. gondii_ infection is most often acquired through the ingestion of sporozoites, encapsulated within sporulated oocysts, or by ingestion of bradyzoite-laden tissue cysts contained within the muscle tissue of an infected animal. Tachyzoites and bradyzoites are resistant to physiological concentrations of trypsin (4), and it has been hypothesized that protease inhibitors may be responsible for this resistance. In _in vitro_ studies here show that recombinant TgPI-1 potently inhibits trypsin and chymotrypsin. Bradyzoite and sporozoite expression of TgPI-1 is the first example of a protease inhibitor expressed in a stage of the parasite exposed to the digestive enzymes in the lower intestine. Furthermore, dense granule proteins secreted by bradyzoites and sporozoites (including TgPI-1) would be expected to come into contact with secreted proteases present within the small intestine (trypsin and chymotrypsin) as the parasites traverse the digestive tract. Upon examination of the lifecycle of _T. gondii_, however, there are other potential functions for TgPI-1.

After bradyzoites/sporozoites penetrate the intestinal epithelium, they differentiate into rapidly dividing tachyzoites, which disseminate throughout the body, proliferating within the PV of infected cells (3). Some tachyzoites probably replicate in intestinal epithelial cells shortly after initiation of infection. In this case, soluble TgPI-1 released from the vacuole upon host cell lysis could help protect the parasite from digestive proteases of the gastrointestinal tract before it enters adjacent cells. This protection would provide the parasite an opportunity to proliferate within the intestinal epithelium before disseminating throughout the host organism.

Interestingly, with the exception of the rhoptry protein, TgROP1, which is degraded within hours of invasion, none of the other DG or rhoptry (ROP) proteins is proteolytically modified after secretion into the PV. This suggests that the PV is largely devoid of proteolytic activity. However, tachyzoites express at least two surface proteases (23) and one ROP-derived protease (TgSUB2) that presumably occupy the PV. Thus, TgPI-1 may serve to inactivate parasite-derived proteases in the PV, thereby preventing them from inappropriately processing vacuolar protein substrates. It is also conceivable that host-derived proteases occupy the PV and TgPI-1 might additionally function to neutralize these proteases and prevent them from degrading the vacuolar contents.

Neutrophils are one of the key immune effector cells of the innate immune response that forms the first line of defense against microbial infections. Recent studies suggest that chemokines rapidly recruit host neutrophils to the site of _T. gondii_ infection where these cells play an essential role in controlling the early infection, possibly by secreting interleukin-12 to initiate the type 1 cytokine immune response (37–39). However, despite being capable of deploying a battery of anti-microbial products, including cytokines, proteases, reactive oxygen intermediates, and nitric oxide, neutrophils fail to prevent _T. gondii_ tachyzoites from establishing the early infection. This suggests that the parasite possesses the ability to neutralize or counteract anti-microbial products released by neutrophils. Our observation that TgPI-1 effectively inhibits neutrophil elastase activity suggests that it could contribute to the ability of the parasite to survive the innate immune response and establish long term infection. TgPI-1 secreted by extracellular tachyzoites, as well as any soluble TgPI-1 released from the PV upon host cell lysis, could inhibit proteases secreted by neutrophils recruited to the site of infection. In this scenario, TgPI-1 could act locally as an anti-inflammatory agent, inhibiting neutrophil proteases such as elastase.

SERPINS, the most extensively studied family of serine protease inhibitors, have been shown to influence viral pathogenesis by dictating viral host range, decreasing inflammation and inhibiting apoptosis (11, 40–42). In addition, a member of the Kunitz family of serine protease inhibitors expressed by the hookworm _Ancylostoma ceylanicum_ has been shown to inhibit chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. The _A. ceylanicum_ Kunitz inhibitor (AceKI-1) may protect the parasites during their transit through the digestive system or provide protection against the host immune system (12). However, the natural target proteases for these microbial SERPINS have not been identified. Kazal inhibitors have been used extensively _in vitro_ to study the interactions of serine proteases with their substrates and are known to bind quickly and tightly to their target proteases. Exploitation of this characteristic tight binding may allow for identification of the natural target protease of TgPI-1 _in vivo_ through co-immunoprecipitation studies. Indeed, we have shown here that rTgPI-1 forms a complex with purified trypsin that is stable under relatively stringent immunoprecipitation conditions (1 M Tris, 1% Triton-X-100, 5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 5 mM EDTA). Additionally, the ability to generate gene-knockout mutants in _T. gondii_ should provide additional insight into the function of Kazal inhibitors in _T. gondii_ and maybe provide evidence for functions in other systems.

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