Cysteine proteinases play a major role in invasion and intracellular survival of a number of pathogenic parasites. We cloned a single copy gene, tgep1, from Toxoplasma gondii and refolded recombinant enzyme to yield active proteinase. Substrate specificity of the enzyme and homology modeling identified the proteinase as a cathepsin B. Specific cysteine proteinase inhibitors interrupted invasion by tachyzoites. The T. gondii cathepsin B localized to rhoptries, secretory organelles required for parasite invasion into cells. Processing of the pro-rhoptry protein 2 to mature rhoptry proteins was delayed by incubation of extracellular parasites with a cathepsin B inhibitor prior to pulse-chase immunoprecipitation. Delivery of cathepsin B to mature rhoptries was impaired in organisms with disruptions in rhoptry formation by expression of a dominant negative µ1-adaptin. Similar disruption of rhoptry formation was observed when infected fibroblasts were treated with a specific inhibitor of cathepsin B, generating small and poorly developed rhoptries. This first evidence for localization of a cysteine proteinase to the unusual rhoptry secretory organelle of an apicomplexan parasite suggests that the rhoptries may be a prototype of a lysosome-related organelle and provides a critical link between cysteine proteinases and parasite invasion for this class of organism.

The protozoan, Toxoplasma gondii, is an obligate intracellular parasite that can invade and replicate within any nucleated cell of vertebrate hosts, including humans (1–3). Invasion by T. gondii tachyzoites is mediated by the sequential regulated release of specialized secretory organelles of the parasite including the micronemes, rhoptries, and dense granules (4). In early observations, the penetration of host cells by tachyzoites was enhanced by the addition of partially purified lysosomal enzymes (5). In addition, proteinases have been implicated in host cell invasion in other members of the Apicomplexa such as Plasmodium (6) and Eimeria tenella (7). We now show that a cathepsin B, toxopain-1, is strongly implicated in T. gondii invasion and that infection can be interrupted with specific cathepsin B inhibitors.

Another unusual feature of Toxoplasma is the absence of a morphologically identifiable lysosomal system. In higher eukaryotic cells, acidic cathepsins in lysosomes are important in protein processing and breakdown. The mammalian precursor of cathepsin B is targeted to the lysosomal compartment by mannose 6-P for proteolytic activation (8). Thus, the apparent lack of a lysosomal system in Toxoplasma raises a number of questions regarding cellular proteinase functions within the parasite. The rhoptries are club-shaped organelles located at the apical end of the parasites with no known counterpart outside of the phylum Apicomplexa (9). Although nine rhoptry proteins (ROP1–9)1 have been identified to date (10), a definitive function has only been determined for ROP2, which mediates binding of host mitochondria to the parasitophorous membrane (11). How rhoptry proteins directly contribute to the invasion process is still not clear. T. gondii rhoptry proteins are synthesized as prepro proteins that are subject to proteolytic cleavage to remove the pressequence and the proregion at the N terminus and then processed to their mature forms (11–14). Rhoptries and pre-rhoptries are the only acidified organelles identified in the parasite (15), contain a high lipid to protein ratio (16), and scavenge sterols from the host cell (17). We now show that the T. gondii cathepsin B, toxopain-1, localizes to the rhoptries, and inhibition of cathepsin B activity or disruption of ROP protein targeting leads to mislocalization of toxopain-1 and abnormal rhoptry biogenesis. This is the first evidence for localization and function of any proteinase to the unusual rhoptry secretory organelle of an apicomplexan parasite.

1 The abbreviations used are: ROP, rhoptry protein; TgCP1, T. gondii cysteine proteinase 1; ELISA, enzyme-linked immunosorbent assay; RACE, rapid amplification of cDNA ends; FPLC, fast protein liquid chromatography; FITC, fluorescein isothiocyanate; Boe, t-butoxycarbonyl; mAb, monoclonal antibody; AMC, 4-amino-7-methyl coumarin; MES, 4-morpholineethanesulfonic acid; Z, carboxyl; HA, hemagglutinin; PBS, phosphate-buffered saline.

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EXPERIMENTAL PROCEDURES

Reagents—All reagents were purchased from Sigma Chemical Co. unless otherwise specified. All AMC and synthetic AMCA peptide substrates were obtained from Enzyme System Products (Livermore, CA). Synthetic peptide inhibitors were a gift from Prototek Inc. (Dublin, CA). Parasite and Host Cell Cultures—Human foreskin fibroblasts (HFF) were initially cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Irvine Scientific, Irvine, CA) and 2 mM glutamine and maintained subsequently in the same medium with 2% fetal bovine serum. The RH strain of T. gondii was obtained from Dr. John Boothroyd (Stanford University, CA). Tachyzoites of the RH strain were maintained in confluent monolayers of HFF cells.

Cloning of the Cathepsin B Gene—A cathepsin B family proteinase gene, tgcpl, was amplified from cDNA with primers based on conserved cationic carboxypeptidase sequence (20) on partial Expressed Sequence Tag (EST) sequence data from the Toxoplasma genome project (EST clone: TgEST5393121.1) (19). The lambda phage probe was obtained from Genome Systems (St. Louis, MO) in vivo excised with ExAssist (Stratagene, La Jolla, CA) as helper phage and sequenced. The remaining sequence was obtained by 5′- and 3′-RACE (Invitrogen). Total cellular RNA from T. gondii was isolated using RNAzol reagent and transcribed into single-stranded cDNA using Superscript II reverse transcriptase and tgcpl-specific reverse primer R1 (5′-GAG TCA TCA TAT CTC TCT TGA CG-3′). Excess DNTPs and primer were removed from single-stranded cDNA, and a homopolymeric tail of dCs was added to the end of single-stranded cDNAs using terminal deoxynucleotidyl transferase. The 5′-end of tgcpl cDNA was then amplified from cDNA using the abridged anchor primer and a specific nested primer 5′-GTC GGG GCC TTC ATG GTC-3′ for 3′-end tailed cDNA using the abridged anchor primer and a specific nested primer F5 (5′-H11032) and the oligo(dT) primer. Finally, the complete gene for T. gondii cathepsin B was amplified by RT-PCR with specific primers against the 5′- and 3′-untranslated sequences, cloned into the TA vector (Invitrogen) and sequenced. All amplified fragments and predicted amino acid sequence compared with known sequences in the data base using the BLASTX, BLASTN, and BLASTP programs at the National Center for Biotechnology Information. Southern hybridization of Toxoplasma genomic DNA with a tgcpl gene probe was performed according to previously described protocols (20).

Homology Modeling of Toxoplasma Cathepsin B—The sequence of toxopain-1 was aligned with human liver lysosomal cathepsin B, rat cathepsin B, and rat cathepsin B. Existing x-ray crystal structures of human liver lysosomal cathepsin B with CA030 inhibitor (PDB ID: 1CSB), human liver lysosomal cathepsin B with no inhibitor (PDB ID: 1HUC), and rat cathepsin B (PDB ID: 1CTX) were used as three-dimensional templates for the modeling of Toxoplasma cathepsin B.

MODELLER, a module of the Quanta software package, was used to generate models of the enzyme.

Expression and Refolding of Recombinant Toxopain-1—To express recombinant enzyme, the sequence of the pro- and mature toxopain-1 was amplified and subcloned into pBAD/Thio TOPO (Invitrogen). The plasmid was transformed into Escherichia coli codon plus cells (Stratagene) in SOB media and induced with 0.02% arabinose with 100 µg/ml kanamycin. The cell pellet was solubilized in 6 M GuanHCl, 0.1 M NaH2PO4, 0.01 M Tris, pH 8.0, 10 mM β-mercaptoethanol, sonicated, and the soluble proteins applied to a nickel affinity column (Invitrogen ProBond), washed with the same buffer at pH 6.3, and eluted at pH 4.5. The protein in the fractions was determined by the Bradford reaction and the fractions screened by SDS-PAGE. One liter of culture yielded 30 mg of the thioredoxin-TgCP1 fusion protein. Thioredoxin-TgCP1 fusion protein (74.5 mg or 1 µg) was loaded to a Nickel-NiNTA (Invitrogen) column, washed with pH 6.0, 30 mM NaCl, 10 mM MgCl2, 10 mM CaCl2, 750 mM arginine, 500 mM GdnHCl, 5 mM reduced glutathione, and stirred in the cold for 48 h. The sample was dialyzed against Tris-buffered saline, pH 7.5 and concentrated to 30 ml. The sample was re-dialyzed against 0.01 M Tris, pH 7.5 and separated by ion exchange FPLC on a Mono Q column.

Proteinase Activity—Proteinase activity in column fractions or tachyzoite lysates were measured by cleavage of peptide substrates. T. gondii RH strain tachyzoites were harvested from human fibroblast monolayers, purified by filtration through 3.0-µm nucleopore filters (Costar, Cambridge, MA) and resuspended at 106 cells/ml in 50 mM Tris buffer, pH 7.4. Following three freeze-thaw cycles and a 15-s sonication, the proteinase activity was measured in the soluble fraction. Substrate specificity was tested for the liberation of the fluorescent leaving group, AMC, from the synthetic peptide substrates to determine the preferred cleavage of the P1 and P2 sites (21). The initial rate of substrate hydrolysis (nmol/min/mg protein) is based on the rate of increase of fluorescence using a Labystems Fluoroscan Spectrofluorometer.

The inhibitor profile (IC50) was determined by monitoring inhibition of hydrolysis of Z-Arg-Arg-AMC substrate in the presence of serial dilutions of the inhibitors following incubation for 30 min at room temperature. The IC50 was calculated as the concentration of inhibitor resulting in 50% inhibition of proteinase activity compared with non-inhibited controls. The peptidyl ketone inhibitors, PRT2005 and PRT2253 (kind gift from Prototek), with a Phe-hPhe substitution in the P2 and P1 residues, were used in all subsequent inhibitor studies.

Antibody Production—Recombinant toxopain-1 was expressed in E. coli and purified by nickel-nitrotriacetic acid affinity column chromatography as above. Polyclonal antibody to recombinant toxopain-1 was produced by immunizing rabbits three times with 100 µg of recombinant protein mixed with Titermax Gold Adjuvant (Sigma). The anti-serum was affinity-purified by adsorption and desorption to recombinant toxopain-1 on nitrocellulose membranes as described previously (22).

Monoclonal antibodies were produced previously by immunizing BALB/c mice with 50 µg of recombinant ACPI (amebic cysteine proteinase 1) using RIBI adjuvant and boosted once intravenously (23). The first antibody harvested and fused with NSO myeloma cells. Three monoclonal antibodies, 1.7, 1.15, and 1.17 were subsequently found to cross-react with T. gondii lysates by immunoblots and ELISA.

Effect of Cysteine Proteinase Inhibitors on Invasion by T. gondii—To evaluate the effect of specific proteinase inhibitors on invasion, tachyzoites (5 × 104 to 1 × 106) were preincubated 30 min at 37 °C in medium alone or containing 20 µM PRT2005 or PRT2253. They were then added directly to fibroblast monolayers on chamber slides (Lab Tek, Nunc) for 2 h. Following washing, the cells were fixed in 4% formaldehyde for 10 min, stained with acridine orange (5 µg/ml), and the number of infected cells was determined by fluorescent microscopy.

Immunofluorescence Microscopy—HFF cells were grown overnight on coverslips and infected with 2 × 105 tachyzoites per well for 24–48 h. Monolayers were washed with phosphate-buffered saline (PBS) and fixed for 15 min in 3% paraformaldehyde (w/v) in PBS at room temperature. Cells were washed in PBS, permeabilized for 5 min in 0.25% Triton X-100 in PBS, and washed three additional times in PBS. Cells were incubated for 1 h with primary antibody diluted in PBS with 3% bovine serum albumin using anti-ROP2/3/4 mAb T3 4A7 (14) or anti-hemagglutinin (HA) epitope tag monoclonal antibody for overexpression of TgCP1 and TgHC. Horseradish peroxidase-conjugated anti-mouse F(ab′)2 IgG (Sigma) and donkey anti-rabbit IgG (Amersham Biosciences) were incubated with FITC-conjugated secondary antibodies and washed in PBS. The slides were mounted and examined by epifluorescent microscopy.

Processing of Rhoptry Proteins—To assess the effect of proteinase inhibitors on rhoptry protein processing in vivo, purified tachyzoites (2.5 × 104) were washed in prewarmed methionine/cysteine-free (Met/Cys-) Dulbecco’s modified Eagle’s medium and incubated for an additional 30 min at 37 °C in medium alone or containing 20 µM inhibitor PRT2253S. Tachyzoites were pulse-labeled with 300 µCi/ml of [35S]methionine/[35S]cysteine (PerkinElmer Life Sciences, Boston, MA) for 15 min in the presence or absence of 20 µM PRT2253S and chased in complete medium for 0–60 min before harvesting. Tachyzoites were washed with PBS containing 1 mM MgCl2 and 1 mM CaCl2, with inhibitor and lysed in 1% SDS, and the radiolabeled rhoptry proteins were immunoprecipitated with mAb T3 4A7 and protein A-Sepharose 4B beads (Amersham Biosciences). The beads were separated by 12% SDS-PAGE, the gel impregnated with fluorographic enhancement solution (Amplify, Amersham Biosciences), dried, and exposed to x-ray film for autoradiography.

Tgα1 Mutants—Tgα1 mutants were produced by PCR with an HA epitope tag introduced into Tgα1 between codons 231 and 232. 2 The resulting D176A mutation had been shown to alter the binding of the tat1-1 mutant tachyzoites (25). The mutated Tgα1-HA(D176A)2 were ligated into pNTP/Sec for transient transfection and into pDHFR-TS/CSM2M3 for stable transfection as previously described (24).

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Conform closely to the T-stretches found in many *T. gondii* genes. The N-terminal 34 amino acids of the prepeptide region possess a putative signal sequence consisting of hydrophobic amino acids. The putative cleavage site between the signal peptide and prepeptide region is predicted by the (−3, −1) rule using the SignalP algorithm of von Heijne (26).

The promodern of toxopain-1 is much longer (239 amino acid residues) and more diverged than those of other cathepsin B family proteases. Toxopain-1 lacks the ERFNIN motif in its promodern, which is found in cathepsin L and H but not cathepsin B. The promodern of toxopain-1 contains two potential sites for asparagine-linked glycosylation NXS/T at residue positions −5 and −186 (27). The mature enzyme (296 residues, \( M_r = 32,362 \)) showed significant similarity to the cathepsin B proteases of *Schistosoma japonicum* (61%) (28), *Sarcophaga peregrina* (59%) (29), and human (60%) (30) (Fig. 1).

Like human cathepsin B, toxopain-1 contains the active site triad of cysteine, histidine, and asparaginate at positions 30, 211, and 231, respectively. Toxopain-1 has 12 Cys residues at similar positions to those of human cathepsin B to participate in formation of 6 disulfide bridges. In cathepsin B family proteases, there is a conserved motif Gly-Cys-Asn-Gly-Gly (residues 73–77). Toxopain-1 has an intact occluding loop (His116 and His117) and likely possesses exopeptidase activity in addition to its endopeptidase function. Unlike the cysteine proteases of *Trypanosoma brucei* and *Trypanosoma cruzi*, toxopain-1 has a much shorter C-terminal extension (−30 residues) (31).

Southern blot hybridization of parasite genomic DNA with a toxopain-1 probe revealed two bands of \( 5200 \) and \( 3000 \) base pairs when cut with *EcoRI* and a single band of \( 19,000 \) base pairs when restricted with *HindIII* and *BglII*.

**RESULTS**

**Cloning of tgep1**—A full-length cathepsin B gene, *tgep1*, was amplified from RH tachyzoite cDNA based on homology to conserved sequences of eukaryotic cysteine proteinases and a partial sequence in the *T. gondii* EST data base (19). The completed transcript of 3236 nucleotides encodes a preprocathepsin B precursor of 569 amino acids (\( M_r = 62,165 \)), comprising a predicted N-terminal signal sequence, a prepeptide domain, and mature enzyme (Fig. 1). The predicted Met start codon conforms to a Kozak eukaryotic consensus sequence (AN-NATGG) for initiation of translation. The sequences leading the ATG start codon (TTTTTTCTACCAAGGAAAAATAGG)
essential His$^{110}$-His$^{111}$ moiety in the loop provides positively charged anchors for binding of dipeptidyl carboxypeptidase substrates for the exopeptidase activity, which is only present in cathepsin B.

**Expression and Purification of Active, Recombinant Toxopain-1**—To express recombinant protein for refolding, we amplified the region encoding the promature sequence and subcloned the fragment into the pBAD/Thio vector, which plasmidified the region encoding the promature sequence and subcloned the fragment into the pBAD/Thio vector, which optimizes expression of soluble protein and allows purification by nickel affinity chromatography of 32-kDa protein consistent with the mature, processed enzyme. A Coomassie-stained gel of the unfolded pro-toxopain-1 and the purified cathepsin are shown. Size markers in kDa are on the left.

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We compared the substrate specificity of the soluble cysteine proteinase activity in tachyzoite lysates to the recombinant enzyme. Substrate specificity was tested by cleavage of synthetic peptide substrates containing Boc-X-X-4-amino-7-methylcoumarin, where X is a positively charged or neutral amino acid residue, to assess the preferred cleavage of the P1 and P2 sites. The initial velocity of cleavage of Z-Arg-Arg-AMC (modeling the specificity of cathepsin B) was greater than seven times that of Z-Phe-Arg-AMC (substrate of cathepsin L) (Fig. 4).

**Cysteine Proteinase Inhibitors Block Toxopain-1 and Parasite Invasion**—Ten new peptidyl ketone cysteine proteinase inhibitors (from Prototek) were screened for their activity against cysteine proteinases in *T. gondii* lysates by measuring the IC$_{50}$. The inhibitors are di- and tripeptides bearing activated ketones, which are irreversible and covalently modify the enzyme. Seven inhibitors were active at concentrations less than 100 μM (data not shown). PRT2005 and PRT2253, two of the most active inhibitors with an IC$_{50}$ < 1 μM were used in the subsequent studies.

To evaluate the effect of inhibitors on host cell invasion, tachyzoites ($5 \times 10^5$ to $1 \times 10^6$) were incubated in medium alone or containing 20 μM PRT2005 and allowed to invade fibroblast monolayers in chamber slides for 2 h. The slides were washed, fixed in formaldehyde for 10 min, and stained with acridine orange for counting. The number of infected cells was significantly less in the presence of the inhibitor ($p < 0.05$ by Student's paired t test, Fig. 5).

**Cathepsin B Localizes to Rhoptries**—To localize toxopain-1, we performed confocal fluorescent microscopy with monoclonal and polyclonal antibodies against toxopain-1, monoclonal antibodies against rhoptry proteins 2, 3, and 4 (14), polyclonal antibodies generated against ROP2 (33), and monoclonal antibodies against micronemes and dense granules. Studies of infected monolayers revealed that toxopain-1 localized to the rhoptries and residual body, but not to micronemes and dense granules (Fig. 6). Cryoimmunoelectron microscopy using affinity-purified polyclonal antibodies to toxopain-1 confirmed that toxopain-1 is specifically localized (90% of labeled gold particles) to the rhoptries, endosomal vesicles (34), and secreted into the parasitophorous vacuolar space (Fig. 7). Toxopain-1 was concentrated in a narrow strip down the center of the rhoptry, in contrast to other rhoptry proteins, which are diffusely distributed.

**Cysteine Proteinase Inhibitors Disrupt the Enzymatic Processing of ROP2**—Because rhoptry proteins are processed prior to their delivery to mature rhoptries (25), we examined whether ROP2 processing is inhibited by specific cathepsin B inhibitors. Processing of pro-rhoptry proteins, ROP2, 3, 4 to mature rhoptry proteins was delayed >60% following preincubation of extracellular parasites with 20 μM PRT2253S for 15 min at room temperature prior to pulse-chase experiments (Fig. 8).

**Disruption of Tgμ1 Function Missorts Cathepsin B to Abnormal Rhoptries**—Localization of *T. gondii* ROP2 is dependent on a tyrosine motif (YEQL) in the cytoplasmic tail that binds to the μ1-chain of the *T. gondii* AP-1 clathrin adaptor (Tgμ1) (24). Tgμ1 adaptin is essential for normal biogenesis of rhoptries (24). A Tgμ1(D176A)-HA mutant with a mutation at a residue necessary for binding to the tyrosine motif functions as a dom-

**Fig. 3. Purification of active recombinant TgCP1.** Pro-toxopain-1 was purified from *E. coli* as a thioredoxin fusion (74.5 kDa). Following refolding and purification, proteolytic fractions contained a 32-kDa protein consistent with the mature, processed enzyme. A Coomassie-stained gel of the unfolded pro-toxopain-1 and the purified cathepsin are shown. Size markers in kDa are on the left.

**Fig. 4. Substrate specificity of *T. gondii* cysteine proteinase.** The cysteine proteinase activities in lysates of purified *T. gondii* tachyzoites (black bars) and active recombinant proteinase (gray bars) were measured with synthetic peptide substrates Boc-X-X-4-amino-7-methylcoumarin, where X is positively charged or neutral peptides. Values are shown as nmol/min/mg and represent the mean ± S.E.
Cathepsin B is critical for Toxoplasma gondii Invasion

Tachyzoites Added X 10^4

FIG. 5. Effect of cysteine proteinase inhibitors on host cell invasion. Tachyzoites (2.5 × 10^4 to 1 × 10^5) were preincubated in medium alone or containing 20 μM of the specific inhibitor, PRT2253, and then allowed to invade fibroblast monolayers in chamber slides for 2 h. The monolayers were washed, fixed in formaldehyde, and stained with acridine orange. Significantly fewer fibroblasts were invaded in the presence of cysteine proteinase inhibitors (black bars) than control monolayers in medium alone (white bars).

FIG. 6. Localization of ROP2 and cathepsin B (TgCP1) by immunofluorescence microscopy. A, toxopain-1 was detected in infected monolayers with mAb CP1.7A and B, ROP2 with mAb T3 4A7. The corresponding phase image is shown in C. Similar colocalization of toxopain-1 and ROP2 to the rhoptries was also observed using the ROP2 polyclonal antibodies (data not shown).

FIG. 7. Immuno-electron microscopy localization of toxopain-1 and ROP2. Monolayers were infected for 24 h, fixed for cryosectioning, and labeled with affinity-purified polyclonal antibodies against TgCP1. A and B, labeling of sections from a cell indicating specific labeling of toxopain-1 to rhoptries. Arrows indicate rhoptry regions that are demonstrated in insets at higher magnification. Bar = 500 nm. C–E, toxopain-1 is also localized to large endosomal vacuole (arrows, C and D) and the parasitophorous vacuole space (arrow, E). Bar = 100 nm.

FIG. 8. Cysteine proteinase inhibitors block processing of pro-ROP proteins. RH tachyzoites were pulse-labeled for 15 min with [35S]methionine/[35S]cysteine in the presence or absence of 20 μM of the specific inhibitor, PRT2253F. Cells were washed and chased for 0–60 min in the presence or absence of inhibitor before immunoprecipitation with mAb T3 4A7, which reacts with ROP2, -3, and -4. The immunoprecipitates at the indicated time intervals were analyzed by SDS-PAGE and fluorography. Total labeled protein is shown in the left lane. Mature ROP2 is shown with an arrow.

Discussion

Cysteine proteinases are critical to invasion by a number of protozoan parasites, including Plasmodium (6) and Eimeria (7). Therefore, we evaluated the role of cysteine proteinases in the pathogenesis of toxoplasmosis. We first cloned a full-length cysteine proteinase gene by amplification from parasite cDNA by 3'- and 5'-RACE based on conserved sequences of eukaryotic cysteine proteinase gene by amplification from parasite cDNA by 3'- and 5'-RACE based on conserved sequences of eukaryotic cysteine proteinase gene (Fig. 1). Cathepsin B, cathepsin D, and human cathepsin B share 44–46% identical to human (30) and Schistosoma (28) cathepsin B family proteinases (Fig. 1). Toxopain-1 contains an occluding loop sequence used previously to identify cathepsin B, conferring exopeptidase activity (Fig. 1) (30) and is present as a single-copy gene.

Homology modeling for toxopain-1 reveals that the overall fold of model structures is very similar to human liver lysosomal cathepsin and rat cathepsin B, particularly at the active sites (Fig. 2). Both of the Toxoplasma and human cathepsin B enzymes have a glutamic acid in the base of the S2 pocket, suggesting a substrate preference for positively charged amino acid residues in the S2 binding pocket.
acids (Fig. 2). To obtain active, recombinant enzyme, we expressed the proenzyme as a thioredoxin fusion protein in E. coli. A number of refolding protocols were evaluated (32), and the resulting purified proteolytically active fractions contained a 32-kDa band consistent with the mature enzyme. The refolded enzyme, therefore, is capable of autocatalytically removing both the prosegment and the thioredoxin fusion (Fig. 3). The preferred substrate of toxopain-1 is for positively charged amino acids, particularly arginine, in the P1 and P2 positions, consistent with cathepsin B activity. The substrate specificities of purified rttoxopain-1 and tachyzoite lysates (Fig. 4), as well as the proteolytic activity released into the medium (data not shown) were essentially identical, suggesting that toxopain-1 is the major cathepsin B proteinase species in the parasite. To date, no other cathepsin B has been identified in this family, toxopain-1 shows very little specificity for Phe/Arg-containing substrates but a distinct preference for Arg/Arg substrates. The homology-based models created for the Toxoplasma protease suggest an explanation for the observed kinetic findings. If the coordinates of another rat cathepsin B structure (PDB ID: 1THE), one with a covalently bound inhibitor at the active site, are superimposed on the model structures of TgCP1, the positioning of the structure 1THE has the sequence, Z-arginine-serine-O-benzyl, with Arg in the P2 position. Superimposition upon toxopain-1 models shows that the Arg in the P2 position of the inhibitor would be ideal to form a stabilizing salt bridge with nearby Glu$^{255}$ (Fig. 2). A Phe in P2, however, would find no stabilizing interactions, and as a result would be a less favorable substrate. The sequence of rat cathepsin B (1THE) has a nearby tyrosine at position 75 that could conceivably make constructive stacking interactions with a Phe at the P2 position of a substrate. Toxopain-1 does not have this tyrosine residue, further supporting the observed substrate profile of the enzyme.

It is interesting to note that, in contrast to other proteases of this family, toxopain-1 shows very little specificity for Phe/Arg-containing substrates but a distinct preference for Arg/Arg substrates. The homology-based models created for the Toxoplasma protease suggest an explanation for the observed kinetic findings. If the coordinates of another rat cathepsin B structure (PDB ID: 1THE), one with a covalently bound inhibitor at the active site, are superimposed on the model structures of TgCP1, the positioning of the structure 1THE has the sequence, Z-arginine-serine-O-benzyl, with Arg in the P2 position. Superimposition upon toxopain-1 models shows that the Arg in the P2 position of the inhibitor would be ideal to form a stabilizing salt bridge with nearby Glu$^{255}$ (Fig. 2). A Phe in P2, however, would find no stabilizing interactions, and as a result would be a less favorable substrate. The sequence of rat cathepsin B (1THE) has a nearby tyrosine at position 75 that could conceivably make constructive stacking interactions with a Phe at the P2 position of a substrate. Toxopain-1 does not have this tyrosine residue, further supporting the observed substrate profile of the enzyme.

FIG. 9. Overexpressing dominant negative TgCP1(D176A) altered rhoptry and cathepsin B trafficking. Indirect immunofluorescence of RH tachyzoites overexpressing wild-type (TgCP1-HA) and dominant negative mutant (TgCP1D176A-HA). Parasites were stained with mAb T3 4A7 to the ROP2 and monoclonal antibody mAb CP1.7 to cathepsin B. The corresponding phase image is shown on the right.

FIG. 10. Cysteine proteinase inhibitors alter rhoptry structure. Infected fibroblast monolayers were grown in the presence of media alone (Control) or media containing the cysteine proteinase inhibitor, PRT2005, at a final concentration of 20 μm for 48 h (+Inhibitor), and visualized by electron microscopy. The small, distorted rhoptries (r) and enlarged Golgi (arrow) are shown in the presence of the inhibitor ($\times 25,500$).

The critical link between rhoptry biogenesis and toxopain-1 was also demonstrated in localization studies in ROP2 or transport mutants. ROP2 is targeted to the rhoptries (Figs. 6 and 7), organelles that play an essential but as yet undefined role in the invasion process of parasites. All nine rhoptry proteins are released as prepro proteins and must be processed to the mature proteins. To determine if toxopain-1 was a key enzyme for ROP processing, we first evaluated the effect of specific cysteine proteinase inhibitors on ROP processing in whole tachyzoites. Greater than 60% inhibition of processing of ROP2, 3, and 4 was detected (Fig. 8). The failure to completely block rhoptry processing might reflect incomplete penetration of the inhibitor or the possible requirement of a proteinase cascade. Ahn et al. (35) and Miller et al. (36) recently reported a subtilisin-like proteinase that localized to the rhoptries and might also play a role in ROP processing.

The critical link between rhoptry biogenesis and toxopain-1 was also demonstrated in localization studies in ROP2 or transport mutants. ROP2 is targeted to the rhoptries by an evolutionarily conserved YEQL sequence in its cytoplasmic tail that binds to μ-chain subunits of the adaptor complex (24). In a dominant negative mutant of the μ1-chain of the adaptor complex, toxopain-1 localizes to distorted rhoptries and residual bodies in the parasitophorous vacuole (Fig. 9). Alternatively, when toxopain-1 is inhibited by growing infected monolayers in the presence of cell-permeant cysteine proteinase inhibitors, rhoptries are small and distorted (Fig. 10).

These studies also shed light on the cellular location of ROP protein processing. Earlier pulse-chase experiments found that processing of ROP2, 3, and 4 was completed in ~30 min (14).
and likely occurred in either secretory granules or in rhoptry precursors (36). Soldati et al. (12) found that both brefeldin A and low temperatures blocked processing of ROP1, so the event was likely post-Golgi. Overexpression of a ROP1-Myc fusion suggested that processing occurred in nascent rhoptries of dividing tachyzoites (12). Similarly, processing of ROP2 was proposed to occur within the secretory pathway en route to the rhoptries (11). Earlier experiments and our findings are consistent with processing of ROP1 and 2 in the late secretory pathway or in maturing rhoptries. Lysosomes have not been identified in T. gondii, but the forming and mature rhoptries are the most acidic compartment in the parasite (15). Both the processed rhoptry proteins and toxopain-1 appear to be densely packed in the mature rhoptries, resulting in the intense signals detected by confocal microscopy (Fig. 6) and immunoelectron microscopy (Fig. 7). This is the first evidence for the localization of and the identification of function of any proteinase to the unusual rhoptry organelle. These findings suggest that the rhoptries may be one of the earliest models of a lysosome-related organelle (37). This may also prove to be an important model for rhoptry protein processing in Plasmodium as well. Similar post-translational processing occurs (38), but the exact site and proteinases involved remain to be determined.

Cathepsin B5 of higher eukaryotes are sorted to lysosomes by mannose-6-P receptors (39). Toxopain-1 does not appear to have mannose-6-P despite two potential glycosylation sites in its prodomain (data not shown). McKerrow and coworkers (40) has identified a prodomain motif in the cathepsin Ls of T. cruzi and Leishmania mexicana, which are required for targeting to lysosomes, but similar signals have not been identified in cathepsin B. We also found that toxopain-1 is released extracellularly into buffer independent of synchronized rhoptry release during invasion. The atypical location of toxopain-1 down the center of the rhoptry (Fig. 7, A and B) suggests a potential subcompartment, which might be released constitutively. Alternatively, toxopain-1 was also detected in endosomal vacuoles (Fig. 7C), which may represent an exosomal pathway of protein excretion (41). Precedence for lysosomal proteinases in a vesicle population comes from Soldati’s group and the localization of cathepsin D. The exact signals that target toxopain-1 to the rhoptries or for extracellular release, independently of cell invasion, are under investigation and could lead to important insights into the biogenesis and targeting to a primitive lysosomal system.

The rhoptry organelles are parasite-specific and play a crucial function in host-cell invasion and establishment of the parasitophorous vacuole. Therefore, targeting inhibitors to the unique rhoptries is particularly attractive for blocking parasite invasion and growth. We have now shown that toxopain-1 plays a key role in cellular invasion and thus may be a target for therapeutic intervention. Protease inhibitors are proving to be potent antimicrobial drugs and may provide a strategy to prevent and/or treat Toxoplasma infections. This goal is critically important for toxoplasmosis in which the current optimal therapeutic regimens are toxic, and life-long suppression is required in severely immunocompromised AIDS patients.

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