Cysteine proteases play key roles in apicomplexan invasion, organellar biogenesis, and intracellular survival. We have now characterized five genes encoding papain family cathepsins from *Toxoplasma gondii*, including three cathepsin Cs, one cathepsin B, and one cathepsin L. Unlike endopeptidases cathepsin B and L, *T. gondii* cathepsin Cs are exopeptidases and remove dipeptides from unblocked N-terminal substrates of proteins or peptides. TgCPC1 was the most highly expressed cathepsin mRNA in tachyzoites (by real-time PCR), but three cathepsins, TgCPC1, TgCPC2, and TgCPB, were undetectable in *in vivo* bradyzoites. The specific cathepsin C inhibitor, Gly-Phe-dimethylketone, selectively inhibited the TgCPCs activity, reducing parasite intracellular growth and proliferation. The targeted disruption of *TgCPC1* does not affect the invasion and growth of tachyzoites as TgCPC2 is then up-regulated and may substitute for TgCPC1. TgCPC1 and TgCPC2 localize to constitutive secretory vesicles of tachyzoites, the dense granules. *T. gondii* cathepsin Cs are required for peptide degradation in the parasitophorous vacuole as the degradation of the marker protein, *Escherichia coli* β-lactamase, secreted into the parasitophorous vacuole of transgenic tachyzoites was completely inhibited by the cathepsin C inhibitor. Cathepsin C inhibitors also limited the *in vivo* infection of *T. gondii* in the chick embryo model of toxoplasmosis. Thus, cathepsin Cs are critical to *T. gondii* growth and differentiation, and their unique specificities could be exploited to develop novel chemotherapeutic agents.

The protozoan parasite, *Toxoplasma gondii*, is unique in its significant impact in both developed and developing countries. One-third of the population in the United States is infected with toxoplasmosis, as is up to 90% of the populations in other countries. *T. gondii* causes a serious opportunistic disease in AIDS patients, organ transplant recipients, and newborns who acquire the infection *in utero* (1, 2). After infection, *T. gondii* tachyzoites proliferate and disseminate widely to all organs. In immunocompetent hosts, acute infection is limited, and the actively dividing tachyzoites transform into metabolically inactive bradyzoites, leading to life-long latent infection. During congenital infection or following severe immunosuppression, such as by the human immunodeficiency virus, toxoplasmosis can reactivate, causing fatal infection. The need for new drugs is pressing as current optimal therapeutic regimens include sulfonamides, which should not be used in pregnant women and cause frequent toxic side effects in others.

*T. gondii* tachyzoites can invade any nucleated cell and survive intracellularly in a specialized parasitophorous vacuole. Within the vacuole, the tachyzoites salvage essential nutrients from the host cell, a process likely to involve parasite proteinases for peptides <1300 daltons. Cysteine proteinases play key roles in host-parasite interactions, including host invasion, parasite differentiation, and intracellular survival (3). Cysteine proteinases have been implicated in cell invasion by two other Apicomplexa, *Eimeria* and *Plasmodium*. Proteinases of *Plasmodium* appear to facilitate erythrocyte invasion and rupture by degrading cytoskeletal proteins and provide free amino acids by degrading host hemoglobin in food vacuoles. Specific inhibition of the *Plasmodium* cysteine proteinases causes accumulation of undegraded erythrocyte cytoplasm in the food vacuoles and inhibits multiplication (3–6).

Cathepsin C (also known as dipeptidyl peptidase I, DPPI) is a unique member of the papain family of cysteine proteinases that include cathepsins B, L, K, H, and S. In contrast to the endopeptidase activity of other papain family cathepsins, cathepsin C has primarily exopeptidase activity and sequentially removes dipeptides from the free N termini of proteins and peptides (7–9). Human cathepsin C plays a role in lysosomal degradation of peptides and also functions as a key enzyme in the activation of granule serine proteases in human and murine immune cells (7–11). We recently char-

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ063593–DQ063595.

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acterized the *T. gondii* cathepsin B (toxopxin-1 or TgCPB)² (12) and cathepsin L (TgCPL) proteases. TgCPB localizes to the rhoptry organelles, which are critical to host invasion (12). Based on specificity differences from host enzymes and their critical role in peptide degradation, *Toxoplasma* cathepsin Cs play important roles in tachyzoite growth and differentiation and may be exploited as drug targets in the future.

**EXPERIMENTAL PROCEDURES**

*Reagents*—All reagents were purchased from Sigma unless otherwise specified. All synthetic 4-aminomethylcoumarin (AMC) peptide substrates and commercially available peptide inhibitors were obtained from MP Biomedicals (Irvine, CA) and Bachem Bioscience Inc. (King of Prussia, PA).

*Parasite Isolation*—Primary human foreskin fibroblasts (HFF) were initially cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA), penicillin, and streptomycin (50 µg/ml) and maintained subsequently in the same medium with 2% fetal calf serum.

*Cloning of the Cathepsin C Genes and Expression Profile*—Two cathepsin C family protease genes, TgCPC and -C2, were amplified from *T. gondii* cDNA with primers based on partial expressed sequence tag sequence data and the preliminary contigs from the Toxoplasma genome project. The remaining sequences were obtained by 3′- and 5′-rapid amplification of cDNA ends and cloned into the TA vector and sequenced (12). All nucleotide sequences and predicted amino acid sequences were compared using the BLAST programs at the National Center for Biotechnology Information. Alignment was performed using ClustalW. Signal sequence prediction was done using SignalP, and other structural predictions were performed with software available at ExPASy.

Total RNA from *in vitro* cultured tachyzoites of *T. gondii* was isolated using RNaseasy reagent (Qiagen), treated with DNase, and transcribed into single-stranded cDNA using Superscript II reverse transcriptase and oligo(dT) primer (Qiagen). Serial dilutions of cDNA obtained from a known number of tachyzoites were used to generate a standard curve of tubulin mRNA expression. The relative copy number was normalized to the copies of tubulin mRNA by real-time quantitative transcript analysis.

Total RNA from *in vivo* encysted bradyzoites was purified with TRIzol (Invitrogen), treated with DNase, and reverse- transcribed as described previously (13). Comparative RT-PCR was performed using equal amounts of tachyzoite and bradyzoite cDNAs based on normalization of the amplified cDNA of *T. gondii* housekeeping actin gene. The cDNA products corresponding to other genes were amplified with 50 pmol of each primer in the presence of 1 µl of Clontech Taq mix DNA polymerase (Clontech) in 50-µl reaction volumes. Thermal cycling conditions for 40 cycles were: 1) denaturation at 94 °C for 10 min; 2) denaturation at 94 °C for 45 s followed by the annealing at 50–60 °C for 1 min and 30 s; 3) elongation for 1 min and 30 s at 72 °C; and 4) additional extension was done at 10 min at 72 °C. Primers used in this study were as follows: TgCPB, forward, 5′-accccgagctcggtttg-3′, and reverse, 5′-cattctctctctcttgc-3′; TgCPC1, forward, 5′-gatcttccatcgcgcaggtc-3′, and reverse, 5′-ctctttcttcttcacc-3′; TgCPC2, forward, 5′-gacgagcttggctttg-3′, and reverse, 5′-ctcccgcttctttcacc-3′; TgCPL, forward, 5′-atggcggatgaagaagtgca-3′, and reverse, 5′-catcgccgagagcagcact-3′; and *T. gondii* actin gene, forward, 5′-atggcgcctgaaggtgccca-3′, and reverse, 5′-catcgctcgctggagcact-3′. PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and photographed using UV-light scanner.

*Expression and Purification of rTgCPC1 and -C2*—To express recombinant proteins, the coding sequence of the TgCPC1 and TgCPC2 were amplified and cloned in-frame into the pET-22b(+) expression vector (Invitrogen). Proteins were expressed in *E. coli* as C-terminally His₆-tagged recombinant protein and purified on a nickel-nitrilotriacetic acid-agarose resin column as described previously (12).

*Purification of Native TgCPC Protease*—*T. gondii* RH strain tachyzoites were harvested from human fibroblast monolayers, washed with PBS, and purified by filtration through 0.2-µm nucleopore filters and resuspended at 10⁹ cells/ml in hypotonic buffer (25 mM Tris, 10 mM EDTA, pH 7.0). Following three freeze-thaw cycles in liquid nitrogen, the soluble extract was fractionated on PD10 columns. The proteins were further purified by ion exchange chromatography on a Mono-Q column as described previously (12).

*Assays of Cysteine Protease Activity*—Protease activity was assayed by the liberation of the fluorescent leaving group, Gly-Arg-AMC, in buffers with pHs ranging from 4.0 to 10.0 using 50 mM acetate for pH 4–6 and 50 mM Tris for pH 6–10.
Enzyme and Inhibition Assays—The inhibitor profile of TgCPC was determined by monitoring inhibition of the cleavage of the preferred substrate, Gly-Arg-AMC (4 \mu M), in the presence of serial dilutions of the inhibitors. Inhibitors at various concentrations were preincubated with the enzyme (10 nM) for 5 min prior to addition of the substrate. Enzyme activity was expressed as the percentage of residual activity when compared with an uninhibited control and was plotted versus increasing inhibitor concentrations to calculate the 50% inhibitory concentration (IC_{50}). Leucine-phenylalanine-vinyl sulfone-methyl was a kind gift from Dr. James Powers (14). Inhibitors were prepared as 10 mM stocks in dimethyl sulfoxide and stored at −20 °C.

Parasite Growth Rate—To evaluate the effect of specific protease inhibitors on invasion and subsequent intracellular multiplication, TgCPC1 knock-out mutants or RH tachyzoites (2 × 10^5) were preincubated 30 min at 37 °C in medium alone or medium containing 100 \mu M cathepsin C inhibitor, glycine-phenylalanine-diazomethylketone (GF-DMK). Parasite replication rates were determined by acridine orange staining at 24 h as described previously (12).

Immunofluorescence Microscopy—Parasites were preincubated for 30 min at room temperature in medium alone or containing 50–100 \mu M cathepsin C inhibitor, GF-DMK, before inoculation at a concentration of 2 × 10^5 tachyzoites/well and grown on coverslips containing confluent HFF cells. Cells were fixed and permeabilized as described previously (12). SAG1 antibodies were obtained from Biodesign International (Saco, ME). GRA3 monoclonal antibodies were a kind gift from Dr. Isabelle Coppens, anti-microneme and AMA-1 were from Drs. Peter Bradley and John Boothroyd, and anti-ROP4 was from Dr. Gary Ward. Cells were incubated for 90 min with primary antibody diluted 1:1000 in PBS with 3% bovine serum albumin. After washing three times in PBS, samples were incubated with fluorescein-conjugated anti-rabbit or anti-mouse antibody (diluted 1:2000 in PBS, 3% bovine serum albumin) and processed according to the protocol described previously (12). The slides were mounted with ProLong Gold with and without 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Invitrogen) and examined by epifluorescent microscopy using a Nikon Optiphot fluorescent microscope.

Electron Microscopy—For transmission electron microscopic studies, infected monolayers were grown in the presence of medium with or without inhibitor for 24 h (50–100 \mu M GF-DMK), fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 for 30 min, washed, and postfixed in 1% osmium tetroxide. Fixed samples were embedded in Epon and thin-sectioned for transmission electron microscopy. Images were recorded with a Tecnai 10 transmission electron microscope (FEI Co.).

Generation of a TgCPC1 Knock-out Mutant—A 3-kb genomic DNA corresponding to the 5' and 3'-flanking region of TgCPC1 locus generated by PCR amplification and cloned into the Apal/XhoI sites of upstream and SpeI/SacI sites of downstream of the pminiHXPRT vector, respectively (16). This resulted in the 5'-TgCPC1-miniHXPRT-3'.TgCPC1 targeting construct. To generate TgCPC1 knock-out mutants, 50 \mu g of linearized targeting construction (Apal-digested plasmid) was transfected into 2 × 10^7 tachyzoites of the T. gondii RH(EP)ΔHXGPRT strain by electroporation using a BTX electropulse cell manipulator 600, a charging voltage of 2.2 kV, and a resistance of 48 ohms. After transfection, parasites were inoculated onto confluent monolayers of HFF in a T75 flask. Stable transformants were selected using 25 \mu g/mL mycophenolic acid and 50 \mu g/mL xanthine added to the culture 24 h after transfection. After two rounds of selection, parasites were cloned in 96-well plates containing confluent HFF cells in the presence of drug as described previously (16), and individual clones were expanded into 24-well plates containing HFF cells for DNA isolation. Parasites from a lysed monolayer were suspended in 250 \mu l of DNA STAT-60 for DNA extraction (Tel-Test, Inc., Friendswood, TX). Genomic DNA was analyzed by PCR for gene knock-out using primers 5'-GTC TCG TTC GCA ACT ACT CGC ACT-3'(c1ko-1; sense) and 5'-CAC CTT TCT TGC ACG GGC ATG CA -3' (c1ko-2; antisense), according to the following program: 94 °C for 30 s; 55 °C for 1 min; 72 °C for 2.5 min for 35 cycles.

Antibody Production and Immunoblots—Polyclonal antibodies to recombinant TgCPCs were produced by immunizing rabbits three times with 100 \mu g of purified recombinant protein mixed with Titermax Gold adjuvant (Sigma). The antisera was affinity-purified by adsorption over a protein G column (Amersham Biosciences) (12). The antibodies to TgCPC1 and -2 did not cross-react. TgCPC1 and TgCPC2 were immunode-tected using rabbit specific polyclonal antisera by SDS-PAGE and Western blotting. Color substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate buffer solution (Zymed Laboratories Inc., South San Francisco, CA) or an ECL kit (Amersham Biosciences) were used for signal detection, and the intensities of the bands were determined with Image software from the National Institutes of Health.

Processing of \beta-Lactamase Protein (BLA)—To construct a plasmid for transient expression of BLA secretion reporter protein, a fragment encoding BLA was amplified from pBluescript, cloned into the plasmid pNTP/sec vector (a kind gift from Dr. Keith Joiner), and used to transfect the HXGPRT-deficient mutant of T. gondii RH tachyzoites as described previously (17). To assess the effect of a cathepsin C protease inhibitor on BLA protein processing in vivo, monolayers of fibroblasts in T25 flasks were infected with RH (BLA) tachyzoites (2 × 10^5) for 24 h at 37 °C in medium alone or containing 100 \mu g GF-DMK (MP Biomedicals). The infected monolayers were then washed three times with PBS and scraped into cold PBS buffer containing Complete protease inhibitors (Roche Applied Science, Mannheim, Germany) plus 100 \mu M E-64. The cell pellet was resuspended in cold 6% trichloroacetic acid for at least 1 h on ice. The precipitates were suspended in SDS sample buffer and analyzed by immunoblot with primary monoclonal anti-BLA antibody (1:1,000 dilution; United States Biological, Swampscott, MA) followed by goat anti-mouse conjugate (Zymed Laboratories Inc.) and developed with an nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate buffer solution (Zymed Laboratories Inc.).

Chicken Embryo Model of Congenital Toxoplasmosis—Fertilized complement fixation for avian leukemia-negative eggs (Charles River SPAFAS, Storrs, CT) were incubated at 37 °C in a humid incubator. To mimic human congenital infection,
tachyzoites (10⁴) were injected directly into the chorioallantoic veins of 12-day-old chicken embryos as described previously (18). The controls included eggs that did not receive injections and embryos that were injected with medium and inhibitor alone (18). The eggs were candled daily to assess viability, and at day 15, the embryos were sacrificed, weighed, and fixed in 10% formalin for histopathological examination. The brain and liver tissues of chicken embryos were frozen at −70 °C for quantitative PCR analysis.

Quantitation of T. gondii Infection By Real-time Quantitative PCR—To quantitate tachyzoites in vivo, the standard curves for SAG1 were used to extrapolate the numbers of tachyzoites present in unknown samples (18). A standard curve was constructed by mixing 10¹⁰ tachyzoites with brain or liver samples (100 mg) from 15-day-old chicken embryos and homogenizing the preparations with a Dounce homogenizer in DNA STAT-60 (Tel-test, Inc., Friendswood, TX). Purified genomic DNA was used as template for PCR amplification to determine the relative amounts of the major T. gondii surface antigen (SAG1) gene by real-time PCR (18).

RESULTS

Expression and Characterization of the cDNAs Encoding T. gondii Cathepsin Cs—We have identified and cloned all three cathepsin C genes from T. gondii. The cDNA sequences encode preproenzymes, each consisting of a signal peptide, a pro region containing two domains that are cleaved, the exclusion and excised prodomains, and a catalytic region (heavy and light chains). The amino acid sequences of the catalytic regions are similar to the mature papain family cathepsins, including cathepsins B and L from T. gondii. Over the entire sequence, the T. gondii and human cathepsin C proteins share 30% identity and 45% similarity (excluding gap positions, Fig. 1). Several residues known to be important for peptidase activity are conserved in TgCPCs: the Asp at the N terminus of the exclusion domain, which blocks the substrate binding cleft beyond the P2 site (7, 8), and Cys, His, and Asn, which form the cysteine protease catalytic triad in the active site (7, 11). A tyrosine residue that binds a chloride ion in the crystal structures of rat and human cathepsin C is also conserved (Tyr-549). Adjacent to the
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active site cysteine, a distinctive tyrosine residue may be involved in the binding of the N terminus of the peptide substrate. This tyrosine motif appears to be unique in the papain family proteases, in which tryptophan usually occupies the position adjacent to the active site residue. The presence of a putative signal sequence at the N terminus of cathepsin Cs suggests that these proteins transit the *Toxoplasma* secretory system. In addition, all *T. gondii* cathepsin C contain a tyrosine-based motif, YXXΦ, where X is any amino acid and Φ is an amino acid with a bulky hydrophobic side chain, and/or an acidic cluster dileucine motif (TgCPC2) located in their cytoplasmic tails known to participate in endosomal/lysosomal protein sorting in higher eukaryotes (12, 19). TgCPC2 has a 100-residue C-terminal extension.

Expression of Cathepsins in Tachyzoites and Bradyzoites—The transition from rapidly replicating tachyzoites to quiescent bradyzoites is one of the most important in the parasite life cycle. We first quantified the relative expression of the different cathepsins in tachyzoites using real-time quantitative PCR. Specific primers were designed to sequences in β-tubulin, a tachyzoite-specific gene (SAG1), and cathepsins B (TgCPB), L (TgCPL), C1 (TgCPC1), C2 (TgCPC2), and C3 (TgCPC3). The copies of mRNA were normalized to the housekeeping gene, β-tubulin (Fig. 2A, TgTub). TgCPC1 is expressed at high levels in tachyzoites, ~20 times that of the TgCPC2 mRNA (Fig. 2A). We estimated that TgCPC1 mRNA was 64% of the total CP mRNA, TgCPB was 21%, and TgCPL was 7% of the total CP mRNA in tachyzoites (Fig. 2A). In contrast, TgCPC3 mRNA was below the level of detection in tachyzoites, and the corresponding expressed sequence tags are only found in partially sporulated oocyst cDNA. Thus, TgCPC3 was not further investigated in this study.

We next determined the transcriptional expression of cathepsin genes using homogenous mature cysts isolated from brains of chronically infected mice. Using RT-PCR, we confirmed that the level of TgCPC1 transcripts in the tachyzoite is dramatically higher than that of TgCPC2 (Fig. 2B). Furthermore, we demonstrated that TgCPC1, TgCPC2, and TgCPB mRNAs cannot be detected in the *in vivo* bradyzoites. TgCPL levels were unchanged between tachyzoites and bradyzoites, similar to the housekeeping actin control (Fig. 2B) Because TgCPC1, TgCPC2, and TgCPB mRNAs are exclusively detected in tachyzoites, these data suggest that the cathepsin genes are stage-specifically down-regulated.

Purification and Catalytic Properties of *T. gondii* Cathepsin C—Attempts to refold recombinant TgCPC1 and -C2 as well as expression of active enzyme in Pichia were unsuccessful, suggesting that another parasite proteinase may be required for the final cleavage of the heavy and light chains. Therefore, we purified native enzyme from tachyzoites. The soluble tachyzoite extracts were fractionated over consecutive hydrophobic, anion exchange, and gel filtration columns. The resulting cathepsin C was 80% pure and reacted with TgCPC1-specific antibodies by Western blot analysis (Fig. 3). TgCPC activity was assayed by the hydrolysis of unblocked dipep-

![FIGURE 2. RT-PCR analysis of cathepsin expression. A, the copies of mRNA of TgCPB, TgCPL, TgCPC1, and TgCPC2 were determined in tachyzoites (as described under "Experimental Procedures") and normalized to β-tubulin (TgTub) as 100. B, semiquantitative RT-PCR was performed to determine the expression of cathepsin genes in tachyzoites cultivated in vitro and mature, encysted bradyzoites isolated from brains of chronically infected mice. The housekeeping actin gene was used as a control to ensure that equal amounts of cDNAs from tachyzoite and bradyzoites were added. The transcripts of TgCPC1, TgCPC2, and TgCPB are exclusively detected in tachyzoites, whereas TgCPL is found in both tachyzoites and bradyzoites.

![FIGURE 3. Expression of recombinant TgCPC1 and TgCPC2 proteins and purification of native TgCPC1. Expression of recombinant mature (lane 1) and full-length (pro + mature domains) (lane 2) proteins is shown. A, 10% SDS-PAGE Coomassie Blue-stained gels and Western blot probed with anti-TgCPC1. B, Coomassie Blue-stained gel and Western blots probed with anti-TgCPC2 antibody. C, native TgCPC1 purified from tachyzoite lysates by fast protein liquid chromatography, electrophoresed by 12% SDS-PAGE, and stained with Coomassie Blue under reducing condition (lane 1) and Western blot detected with TgCPC1 antibody (lane 2) The arrows indicate TgCPC1 subunits. There was no cross-reaction with anti-TgCPC2 antibody (data not shown).
tide substrates, H-Gly-Phe-AMC, H-Gly-Arg-AMC, two mammalian cathepsin C substrates, and H-Arg-Arg-AMC, a substrate for DPP III activity. To determine endopeptidase activity, we also tested N-terminal blocked dipeptide AMCs, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC, preferred substrates for the endopeptidases cathepsins B and L, respectively. We found that T. gondii cathepsin C did not have endopeptidase activity and did not cleave either the blocked substrate Z-Phe-Arg-AMC or the blocked substrate Z-Arg-Arg-AMC. TgCPC1 does not possess a high affinity for the human cathepsin C-specific substrate GF-AMC but displayed a strong preference for the GR-AMC. The hydrolysis of GR-AMC by cathepsin C was 17-fold higher than that of GF-AMC substrate. T. gondii cathepsin C did not cleave the DPP III (a metallo-exopeptidase of serine protease family)-specific substrate H-Arg-Arg-AMC. TgCPC1 did not cleave mature cysteine proteinases of cathepsin B and L, which contain a proline at the N termini of the P2’ position. These results indicated that TgCPC preferentially hydrolyzed N-terminal peptides with a penultimate basic residue such as Arg, whereas those containing basic amino acids (Arg or Lys) in the ultimate position (e.g. H-RR-AMC), as well as those containing a proline residue at either side of the peptide bond (e.g. mature cathepsin B and cathepsin L), are resistant to attack (Fig. 4). The TgCPC1 was active in a broad pH range of 4.5–8.0, with a pH optimum near pH 6.5, and was significantly stimulated by the reducing agent dithiothreitol.

Inhibitor Profile of T. gondii Cathepsin C—Not surprisingly, the protease activity of TgCPC was not affected by metallo- or serine proteinase inhibitors (data not shown). Two irreversible cathepsin C inhibitors, GF-DMK and leucine-phenylalanine-vinyl sulfone-methyl, had IC₅₀s of 14.8 and 31.3 μM, respectively (9–11). Neither compound inhibited active, recombinant TgCPL or TgCPB (data not shown).

Inhibition of Intracellular Multiplication by Cathepsin C Inhibitor—To evaluate the effect of a cathepsin C inhibitor on intracellular parasite multiplication, RH tachyzoites (2 × 10⁵) were added to fibroblast monolayers on 8-well chamber slides and allowed to invade for 2 h. Free parasites were then washed away, fresh medium containing 100 μM cathepsin C inhibitor (GF-DMK) were added, and the number of tachyzoites per parasitophorous vacuole was determined at 24 h (Fig. 5). Control infection resulted in significantly more tachyzoites/vacuole and more infected cells (p < 0.01 by Student’s t test). At the 24-h time point, 51% of parasitophorous vacuoles of the control parasites contained greater than eight tachyzoites, whereas with the inhibitor, only 17% of the vacuoles had more than eight tachyzoites (Fig. 5). The cathepsin C inhibitor, GF-DMK, produced a concentration-dependent inhibition of parasite growth and replication over the initial 24 h following host cell entry.

To try to identify the sites of inhibitor action in intracellular tachyzoites, we compared the morphology of inhibitor-treated and control parasites by fluorescent and electron microscopy. The finding of fewer parasites/vacuole in inhibitor-treated parasites was confirmed, but no abnormal morphology of parasites or fibroblasts was detected by fluorescent microscopy (Fig. 6) or electron microscopy (data not shown).

TgCPC1 Knock-out Mutant—To investigate the specific physiologic role of TgCPC1, we generated a TgCPC1 knock-out through gene disruption and analyzed the phenotype of the resulting mutants. TgCPC1 is a single-copy gene in the Toxoplasma genome, so one round of gene targeting was sufficient to create null mutants by replacing introns 3–5 of the TgCPC1 locus with the HXGPR gene. Sixty transformants were screened by PCR for the presence of the TgCPC1 locus. Only one clone lacked the 1.9-kb PCR amplified product characteristic of the wild-type locus introns 3–5, which was replaced by the HXGPR gene in a double cross-over event (Fig. 7, A and B).

We compared the ability of both parental and knock-out parasites to invade and replicate in host cells. TgCPC1-deficient parasites caused only a slight delay in parasite replication, and tachyzoites egressed from the host over a time course similar to that of the parental RH strain. Twenty-four hours following invasion of the host cell, there were no significant differences in the number of cells infected or the average number of parasites per vacuole between the ΔTgCPC1 mutants and the wild-type parasite. Because the effect of inhibitors of all TgCPC activity was more dramatic than the effect of the TgCPC1 knock-out, we asked whether TgCPC2 might be up-regulated in these parasites. Indeed, we found that TgCPC2 was up-regulated 4.5-fold in TgCPC1-ko parasites by quantitative PCR analysis (Fig. 7C) and apparently can substitute for TgCPC1 function. Attempts to make a double TgCPC1-TgCPC2 knock-out were unsuccessful.
**Cathepsin Cs Are Key for Intracellular Survival of *T. gondii***

Control Infection

![Control](image)

+ Inhibitor

![Inhibitor](image)

**FIGURE 6. Morphology of inhibitor-treated tachyzoites.** Following infection with tachyzoites for 2 h, the monolayers were incubated with medium alone (A and B) or media containing 75 μM GF-DMK (C and D) for 48 h at 37 °C. Parasites were detected by immunofluorescence staining of the major surface antigen SAG1 (A and C) or by 4′,6-diamidino-2-phenylindole nuclear staining (DAPI) (B and D). Fewer parasites were detected in infected cells treated with the inhibitor, but the parasite morphology was normal. Magnification was ×1000.

Localization of TgCPC—To localize the cathepsin C in tachyzoites, HFF monolayers on chamber slides were infected with the RH strain for 24 h. Following fixation and permeabilization, the cells were incubated with TgCPC1 (Fig. 8) or TgCPC2 antibody (data not shown). TgCPC1 and TgCPC2 are found in the dense granules in tachyzoites of *T. gondii* (Fig. 8), but not rhoptries or micronemes (data not shown).

*TgCPC Degradates Proteins in the Parasitophorous Vacuole (PV) of *T. gondii*—Because cathepsin Cs of Plasmodium falciparum play a role in degrading hemoglobin, we evaluated whether *Toxoplasma* cathepsin Cs played a similar role in degrading peptides. We used transgenic parasites that expressed *E. coli* BLA under the control of the NTPase promoter and signal peptide as a marker (17). The foreign protein is transported in dense granules and secreted into the vacuolar space. Once released, the BLA is rapidly degraded in the parasitophorous vacuole (17). When parasites were treated with the cathepsin C inhibitor, GF-DMK, however, degradation of BLA was prevented (Fig. 9). These findings suggest that cathepsin Cs are directly or indirectly required to degrade proteins or oligopeptides in the vacuolar space.

*TgCPC Inhibitors Block In Vivo Infection in Chick Embryo Model—*To test whether the reduction in the parasite growth and multiplication observed by cathepsin C inhibition would have an effect on the parasite virulence in vivo, chick embryos were inoculated with wild-type tachyzoites with or without cathepsin C specific inhibitor or the ΔTgCPC1 mutant. Freshly harvested tachyzoites were inoculated into 12-day-old chick embryos (10^4 parasites/chick), and the number of parasites in the chick brain and liver was determined 3 days later by quantitative PCR based on standard curves for SAG1 as described previously (18). Inhibition of all TgCPC activity by the inhibitor, GF-DMK, significantly reduced the virulence of *T. gondii* in vivo (*p* < 0.01) (Fig. 10). The effect of the TgCPC1 knock-out on in vivo infection was less significant (*p* > 0.05), probably because a compensatory increase in TgCPC2 (Fig. 7C) attenuated the effect.

**DISCUSSION**

Cathepsin C (DPPI) is a structurally and catalytically unique member of the papain family of cysteine proteases. Initially, it was believed to function primarily in intracellular protein degradation and turnover (6–10). However, further studies found that cathepsin C may play a role in cell growth, differentiation, and protease activation (20, 21). In addition, cathepsin C can be secreted into the extracellular compartment and may play a part in extracellular matrix degradation (6–10). These observations suggest more diverse roles for this group of proteases. Cathepsin C can selectively activate granule serine proteases, as demonstrated by findings that cell lines derived from cathepsin C-deficient mice fail to activate groups of serine proteases from granules of immune cells (cytotoxic T-lymphocytes, natural killer cells) and inflammatory cells (neutrophils, mast cells) (10, 11). Activation of the granzymes requires the cleavage of the prodipeptide, which then presumably allows the mature enzyme to fold into a catalytically active conformation. Several recent studies suggest that cathepsin C is capable of performing this processing event with parasitic enzymes in vitro, as demonstrated by its ability to activate a cathepsin B of *Schistosoma mansoni* (21). Studies to detect potential proteinase activation by TgCPCs are underway.

Unlike most protozoan parasites such as *Entamoeba histolytica*, which has more than 40 genes encoding Clan CA cathepsins (22), the repertoire of cathepsins in *T. gondii* is limited with only one cathepsin B, one cathepsin L, and three cathepsin C genes. We have previously shown that TgCPB localizes to the rhoptries and that potent new cathepsin B specific inhibitors or antisense to TgCPB reduced intracellular multiplication (12). We have also expressed active TgCPL and are characterizing its function.

We now test the hypothesis that cathepsin Cs, which are only present in a limited number of human cells, may play a critical role in the pathogenesis of toxoplasmosis and may represent a novel drug target. We have identified three cathepsin C genes, which have ~30% identity with the human gene (Fig. 1). TgCPC1 is the most abundant thiol protease in *T. gondii* tachyzoites (Fig. 2A) and is down-regulated in bradyzoites encysted in vivo (Fig. 2B). The TgCPC3 gene appears to be expressed only in the sporozoite stage.

The mammalian cathepsin C is primarily an amino dipeptidase, which cleaves two-residue units from the N terminus of a polypeptide chain. The enzyme is not very specific and will progressively cleave two-residue units from protein and pepti-
dyl substrates until either the N terminus is no longer available or a stop sequence has been reached. Active human cathepsin C exists as a tetramer, each consisting of a heavy chain (23 kDa), a light chain (7 kDa), and a propeptide (16 kDa) that remains associated (7). In contrast, the other members of the papain family are monomers. We readily expressed recombinant TgCPC1 but were unable to express active enzyme (Fig. 3). Therefore, we characterized the enzymatic specificity of native TgCPC1. Using five synthetic dipeptide substrates, we found a preference for arginine at the substrate P1 position (Fig. 4). Cathepsin C stop sequences are positively charged residues (arginine or lysine) at the N terminus, proline at either side of the scissile bond (P1 or P1'), or isoleucine at P1 position of a substrate. This selectivity provides an opportunity for the design of inhibitors selective for TgCPC, which has a more restricted substrate specificity than previously recognized and underscores the importance of a free amino group at the N terminus of cathepsin C substrates.

We had shown that peptidyl ketone and hydrazide inhibitors were very effective against TgCPB and were concentrated in the parasite and not the host cell, supporting the feasibility of targeting only parasite enzymes (12). The cathepsin C inhibitor, GF-DMK, was shown to readily enter all cells (10), and treatment of human myeloid cells with GF-DMK selectively reduces the generation of enzymatically active serine proteases within the granules of CTL and myeloid cell lines by inhibiting cathepsin C activity (9, 10). GF-DMK inhibits the native TgCPC1 with an IC₅₀ of 14.8 μM.

We next tested GF-DMK for its ability to block the intracellular development of newly invaded Toxoplasma tachyzoites over the first 24 h after entry. Parasite growth and replication was blocked, and fewer parasites were seen in each parasitophorous vacuole (Figs. 5 and 6), although at relatively high concen-
TgCP1 knock-out parasites found that these mutants can still penetrate and replicate in host cells, indicating that TgCPC1 is not essential. Twenty-four hours following invasion of the host cell, we were not able to detect any significant difference in the average number of parasites per vacuole between the ΔTgCPC1 mutants and the wild-type parasite. We did find, however, that TgCPC2 was up-regulated in the mutants, which suggests that it may perform a similar function. The compensation was not complete, however, as ΔTgCPC1 parasites still had attenuated virulence in the chick in vivo model of toxoplasmosis (Fig. 10).

Attempts to generate double disruption mutants for TgCPC1/TgCPC2 were unsuccessful, suggesting that at least one of these two proteases is required for host cell invasion and growth in *T. gondii* tachyzoites. Unexpectedly, none of the TgCPC mRNAs were detected in mature encysted bradyzoites isolated from brains of chronically infected mice, suggesting that these cathepsin functions may not be required for bradyzoite growth. In contrast, TgCPC3 appears to be exclusively expressed in the sporozoite stage and may play an important role during primary infection. In the future, it would be interesting to generate TgCPC3 knock-out mutants in a strain that is capable of differentiating into bradyzoites and sporozoites to study these interactions.

*T. gondii* is an obligate intracellular parasite that resides inside a PV derived from the host cell membrane upon invasion but is rapidly modified by the parasite. Release of proteins from secretory dense granule organelles is one of the major processes developed by the parasite to remodel its newly formed niche into an active compartment that remains segregated from the host cell endocytic machinery. Most of the dense granule proteins are predicted transmembrane proteins that are secreted into the vacuolar space as soluble proteins. One of the current hypotheses about their function is that they could be implicated in the exchanges established between the parasite and its host cell such as acquisition of nutrients, signal transduction, or maintenance of the PV in the host cell cytoskeleton, allowing successful parasite development (24). TgCPC1 and TgCPC2 localize to dense granules (Fig. 8), similar to the localization of *P. falciparum* cathepsin C, which participates in the breakdown of hemoglobin (6). We showed that inhibition of TgCPC1 and TgCPC2 blocked the breakdown of a marker secreted protein, β-lactamase, in the PV (Fig. 9). The dipeptides generated by TgCPCs are likely hydrolyzed to amino acids by cytosolic neutral aminopeptidases. Indeed, aminopeptidase homologs have been identified from expressed sequence tag data, and contigs have been identified from the *T. gondii* genome sequence (ToxoDB: the Toxoplasma gondii Genome Resource). Not surprisingly, inhibition of TgCPC by peptide inhibitors (Fig. 5) or knock-out of TgCPC1 decreased parasite multiplication in vivo (Fig. 10).

In summary, cysteine proteases play central roles in apicomplexan invasion, organellar biogenesis, and intracellular survival (3). The endopeptidases, cathepsins B and L, have been shown to play important roles in the pathogenesis of *T. gondii* (12).³ Cathepsin C is an exopeptidase, which we have shown is

³ X. Que, R. Huang, S. Herdman, J. C. Engel, and S. L. Reed, unpublished data.
involved in the breakdown of peptides in the PV. In humans, cathepsin C activates granule-associated serine family proteases, and we are investigating a similar role in activating Toxoplasma proteinases. Because the distribution of cathepsin C is limited in human cells, and we have shown that inhibition of this class of enzymes results in significant attenuation of infection by Toxoplasma, specific inhibitors may prove to be novel therapeutic agents.

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