Cathepsin B Homologue at the Interface between a Parasitic Nematode and Its Intermediate Host

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Parelaphostrongylus tenuis is a parasitic nematode that causes a debilitating neurologic disease in many North American cervids and domestic livestock species. We produced a PCR-based cDNA library from infective larvae (L3) in order to identify molecules that mediate parasitism. A dominant 1,250-bp amplicon encoded a homologue of cathepsin B cysteine proteases. The sequence incorporated a C29G substitution in the putative active site. Antibodies generated against a recombinant form detected the native protein (PtCPR-1) in Western blot assays of L3, but not adult worm, extracts. Immunohistochemical methods revealed that PtCPR-1 synthesis was restricted to larval stages within the snail intermediate host (*Triodopsis* sp.), beginning as early as 2 days postinfection (dpi) of snails. The protein was present in the intestine and luminal contents and was lost from larvae over time. Concurrent studies showed that larvae induced an immune response in snails beginning at 1 dpi. Layers of hemocytes encapsulated larvae immediately after infection, and granuloma-like structures formed around parasites in chronic infections. Loss of PtCPR-1 from L3 and its accumulation in host tissues coincided with degeneration of granuloma architecture 90 to 105 dpi. Fully developed L3 emerged from the snail at this time. Our data implicate PtCPR-1 in larval development and possibly in the emergence of *P. tenuis* from the intermediate host. Emerged L3 survived desiccation and cold stress, suggesting that they could remain infectious in the environment. Molecules promoting emergence would facilitate dispersal of L3 and increase the likelihood of transmission to definitive hosts.

Invertebrates serve as vectors and intermediate hosts for many parasites of animals and humans. As such, they play a pivotal role in the transmission and dissemination of pathogens. Host immunity is an important influence in parasitic infection. Evasion of immunity is crucial to the survival of parasites that require prolonged association with their hosts. Although invertebrates lack an adaptive immune system, they possess innate defenses mediated by hemocytes and soluble hemolymph factors (30). In gastropods, the principal reaction to tissue invasion is an aggregation of hemocytes that encapsulates metazoan parasites (3, 31).

Innate responses in gastropods are best characterized in snails infected with the digenetic trematode *Schistosoma mansoni*. Hemocytes from resistant snail species encapsulate and kill the parasite, whereas hemocytes from susceptible snails fail to perform either function (3). In contrast, nematodes that utilize gastropods as intermediate hosts (superfamily *Metastomylidae*) are encapsulated yet survive in susceptible host species (18, 37). This suggests that nematodes and trematodes may employ different strategies to evade immunity in their intermediate hosts. We sought to investigate nematode molecules that function at the host-parasite interface during infection with *Parelaphostrongylus tenuis*, a neurotropic parasitic nematode of white-tailed deer in eastern North America. Infection in white-tailed deer is asymptomatic; however, in atyp-

ical hosts, including sheep, goats, llamas, and alpacas, the worm causes neurologic disease when it migrates in the parenchyma of the central nervous system (27). Terrestrial gastropods serve as intermediate hosts, supporting the development of third-stage larvae (L3) that initiate infection in vertebrate hosts (1). We prepared a reverse transcription (RT)-PCR-based cDNA library from L3 of *P. tenuis* and found that the dominant cDNA encoded a homologue of cathepsin B cysteine proteases. Protein synthesis was developmentally regulated and restricted to larval stages that developed in the intermediate host. Loss of the protein from L3 and its accumulation in surrounding host tissues coincided with disruption of the architecture of the local cellular infiltrate. As granulomas degenerated, larvae emerged from the gastropod. Our findings suggest a role for cathepsin B-like cysteine proteases in larval development and emergence from the intermediate host.

MATERIALS AND METHODS

Parasites and E/S products. Adult and L1 *P. tenuis* nematodes were collected as described previously (14). L3 were cultured in laboratory-reared terrestrial gastropods (*Triodopsis* sp.) and recovered by digestion (0.6% pepsin, 90 mM HCl). Excretory and secretory (E/S) products were collected from L3 cultured at 37°C in minimal essential medium (Gibco BRL; Grand Island, NY) containing antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco BRL) and 10% heat-inactivated white-tailed deer serum ultrafiltrate (<10 kDa; Centricon YM-10; Fisher Scientific, Pittsburgh, PA). Adult E/S products were collected previously (13).

Cloning and sequencing of the dominant L3 cDNA. Total RNA was extracted from 86 *P. tenuis* L3 recovered at 72 to 74 days postinfection (dpi) as described previously (14). Double-stranded cDNA was synthesized by RT-PCR (SMART cDNA Library Construction Kit; Clontech, Palo Alto, CA), digested with SfiI, purified with Sephacryl S-400 (Promega, Madison, WI), cloned into the λTri-

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FIG. 1. Detection of *Ptcp-1* and *Ptcp-2* transcripts in different life stages of *P. tenuis* by RT-PCR. (A) The dominant 1,250-bp product (*Ptcp-1*; arrowhead) amplified from L3 cDNA was cloned and identified by sequencing to be a homologue of cathepsin B cysteine proteases. (B and C) Life stage expression of *Ptcp-1* (B) and *Ptcp-2* (C) detected by RT-PCR. PCR was performed on cDNA from the life stages of *P. tenuis* with gene-specific primers spanning a 334-bp amplicon of *Ptcp-1*, a 591-bp amplicon of *Ptcp-2*, and a 200-bp amplicon of a housekeeping gene (*Pthsp-70*). M = adult male; F = adult female; L3 = infective larvae; L1 = first-stage larvae; H = water control; G = genomic DNA control.

pLex2 vector (Clontech), and packaged (Gigapack III; Stratagene, La Jolla, CA). The primary library contained 8×10^5 clones with an average insert size of 650 bp. The library was amplified to 9.6×10^{10} clones.

Double-stranded L3 cDNA was resolved in a 2% agarose gel (SeaKem LE; FMC BioProducts, Rockland, ME), stained with ethidium bromide (0.5 μ g/ml), and detected with UV light (GDS 7500 Darkroom; UVP, Upland, CA). DNA was purified from the dominant 1,250-bp amplicon (Fig. 1A) and cloned into pCRII (Invitrogen, Grand Island, NY). Bacterial colonies were assayed for inserts with T3 and T7 universal primers in PCR. Inserts were sequenced (BioResource Center, Cornell University, Ithaca, NY) with T3, T7, and gene-specific internal primers (cpr-1-F, 5'-GAA AGC AGC AGG TCA TCC TC-3'; cpr-1-R, 5'-GAA GCA TCC CCA ACA AGA AC-3'). Subsequently, PCR was performed on L3 double-stranded cDNA to amplify and validate the full-length sequence (forward, 5'-GAC ACA TAC GCT GCT TCC TG-3'; reverse, 5'-CGC TGA GGT CTT CAC ATC AC-3'). Amplicons were sequenced on both strands with forward and reverse primers.

Sequences were edited, and open reading frames were deduced. Similarity to sequences from nonredundant databases was determined with the basic local alignment search tool (BLAST, version 2.2.11; National Center for Biotechnology Information, Bethesda, MD). Sequences with the highest scores were aligned with the *P. tenuis* sequence (MacVector Version 6.5.3; Accelrys, Princeton, NJ). Similarity to expressed sequence tag sequences was determined with tBLASTn (National Center for Biotechnology Information). A putative signal peptide was identified with SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Potential N-linked glycosylation sites were predicted by the presence of N-X-S/T in the open reading frames (<http://www.cbs.dtu.dk/services/NetNGlyc/>). In order to confirm the dominance of the 1,250-bp product in L3 cDNA, 10 clones from the primary L3 library were selected, sequenced, and analyzed as described above.

RT-PCR. In order to evaluate developmental regulation of the genes under study, double-stranded cDNA was synthesized as described above (SMART system; Clontech) with RNAs prepared from L1, L3, adult male, and adult female *P. tenuis*. Preparations were normalized for cDNA abundance by using as a reference the gene for ubiquitously expressed heat shock protein 70 (*hsp-70*) (14). cDNAs were amplified with gene-specific primers for *Ptcp-1* (334-bp product; cpr-1-F, cpr-1-R), *Ptcp-2* (591-bp product; cpr-2-F, 5'-ATG GTG GGT ACC CGA TCT CT-3'; cpr-2-R, 5'-ATG GTG GGT ACC CGA TCT CT-3'), and *hsp-70* (14) in 30 cycles of PCR (95°C for 30 s, 55°C for 30 s, 72°C for 1 min). Genomic DNA from *P. tenuis* was included in control reaction mixtures to demonstrate introns. Nucleotide sequences of *Ptcp-1* amplicons prepared from cDNA were confirmed by sequencing.

Recombinant protein synthesis and purification. The cDNA encoding the proprotein of *Ptcp-1* was amplified from L3 double-stranded cDNA (Express-F, 5'-GCA TTT CTC CAG CAT ACA GAG-3'; Express-R, 5'-TAG CTC ATT ATT TCG GCT TTC C-3'), cloned into the bacterial expression vector pTrcHis (Invitrogen), and verified by sequencing with vector and internal primers (cpr-1-F and cpr-1-R). The protein was synthesized as a recombinant (rPtCPR-1) with an N-terminal polyhistidine (His) tag for purification under denaturing conditions (8 M urea) by nickel chelation affinity chromatography (Ni-NTA agarose; QIAGEN, Valencia, CA).

Antisera. Three AO strain rats were injected intraperitoneally with 100 μ g of purified rPtCPR-1 or His-tagged recombinant *lacZ* (*rlacZ*) mixed with Freund's incomplete adjuvant (Sigma, St. Louis, MO). After 4 weeks, animals were boosted with 75 μ g of protein in adjuvant by the same route. Blood was collected 6 weeks later, and serum was stored at -20°C .

Rats were housed in the James A. Baker Institute vivarium according to guidelines of the American Association for Accreditation of Laboratory Animal Care.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Adult *P. tenuis* E/S products (150 μ g), adult somatic extracts (150 μ g), and 4,050 L1 or 280 L3 disrupted in 2 $\times$ sample buffer were resolved under reducing conditions in 11% acrylamide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). Proteins were transferred to nitrocellulose membranes (42), and strips were incubated with rat antiserum diluted 1:100 in 5% milk. Blots were incubated with horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (5.4 μ g/ml; ICN Pharmaceuticals, Inc., Aurora, OH) and developed with chemiluminescent substrate (ECL Reagent; Amersham Pharmacia Biotech, Piscataway, NJ) and autoradiography.

Emerged L3 were collected at 90 to 105 dpi to compare the abundance of PtCPR-1 with tissue L3. Tissue L3 were recovered from snails by digestion at 90 to 105 dpi and 135 dpi. Protein extracts (27 L3 per lane) were resolved and blotted as described above. Duplicate blots were developed with antibodies specific for a ubiquitous protein (rPtAPI-1) (14) or for rPtCPR-1.

Histology and immunohistochemistry. Adult *P. tenuis*, L1, and snails infected 0 to 105 days previously were fixed in 10% neutral buffered formaldehyde solution and embedded in paraffin. Sections (5 μ m) were cut and mounted on glass slides, deparaffinized, and either stained with hematoxylin and eosin (H&E) or processed for immunohistochemistry. Antigens were unmasked by incubation in 0.01 M citric acid (pH 6.0) for 40 min at 90 to 95°C (21). Sections were incubated with rat antiserum diluted 1:50 and horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (16.2 μ g/ml) and developed with 3-amino-9-ethylcarbazole (Sigma) as the substrate. Sections were counterstained with Gill's modified hematoxylin (Electron Microscopy Sciences, Hatfield, PA), and coverslips were mounted with Glycergel (Dako Corporation, Carpinteria, CA). Images were captured with an Olympus BX51 microscope fitted with a DP12 digital camera (Olympus, Melville, NY).

Assessment of L3 emergence from snails. Laboratory-reared juvenile *Triodopsis* sp. snails were fed romaine lettuce and carrots and maintained beneath nylon window screening in plastic terrariums with a 12-h daylight cycle. Snails (shell diameter, 5 to 8 mm) were exposed to *P. tenuis* at a density of 100 to 200 L1 per snail as described by Anderson (1). Following exposure, snails were transferred to a clean terrarium every 3 days. Terrariums, window screening, and food were washed with 0.85% saline under agitation on an orbital rotator. Sediment from the saline was examined to count and recover L3.

In the first trial, 44 snails were monitored over 153 days (105 to 191 and 216 to 258 dpi). In the second trial, 30 snails were monitored for 274 days (19 to 293 dpi). At 45, 160, and 293 dpi, five snails were killed and digested to recover and count L3. Larval burdens were compared by one-way analysis of variance.

Emerged L3 were maintained at 4°C in saline or tap water for up to 1 year to assess survival outside of the host. In addition, survival of emerged L3 was assessed following desiccation and freezing. Lettuce from terrariums, harboring unknown numbers of larvae, was air dried for 24 h and then washed to recover emerged L3. In a subsequent trial, lettuce was air dried, stored at -20°C inside a Ziploc bag for 24 h or 7 days, and washed to recover emerged L3.

Nucleotide sequence accession numbers. The nucleotide sequences of *Ptcp-1* and *Ptcp-2* were submitted to the GenBank database and assigned accession no. DQ164840 and DQ164841, respectively.

RESULTS

Analysis of *Ptcp-1* and paralogues. An amplicon of 1,250 bp dominated the L3 double-stranded cDNA (Fig. 1A). A BLASTp search with the deduced amino acid sequence re-

FIG. 2. ClustalW alignment (MacVector 6.5.3) of the deduced amino acid sequences of PtCPR-1, PtCPR-2, and parasitic helminth cathepsin B cysteine proteases. Amino acid positions are numbered along the margins. The overline at the N terminus indicates the predicted signal peptide, shaded areas indicate conserved residues, asterisks indicate residues that make up the catalytic triad, boxed cysteine residues (C) are invariant, predicted N-linked glycosylation sites in *P. tenuis* sequences are indicated by horizontal boxes, and the vertical line marks the putative cleavage site of the proregion from the mature protein. The Cys29Gly substitution in PtCPR-1 disrupts the putative active site. Sequence identities (mature proteins) relative to PtCPR-1 are indicated in the box at the lower right.

vealed 60% similarity to a cathepsin B-like cysteine protease from *Caenorhabditis elegans* (GenBank NP_741818) (28). The parasitic nematode protein with the greatest similarity was a cathepsin B-like cysteine protease from *Ascaris suum* (GenBank AAB40605). Alignments of selected helminth sequences with high identity to the *P. tenuis* sequence are shown in Fig. 2. The gene from *P. tenuis* was designated *PtCpr-1* based on its similarity to cathepsin B cysteine proteases. PtCPR-1 and homologous proteins from other helminths have in common a signal peptide and 13 conserved cysteine residues. In addition, a catalytic triad, composed of cysteine, histidine, and asparagine residues, is highly conserved among helminth cathepsin B cysteine proteases (38). A glycine replaces the active-center cysteine at position 29 of the mature protein (Fig. 2), classifying PtCPR-1 as a nonpeptidase homologue of cathepsin B (MEROPS database resource for peptidases [http://merops.sanger.ac.uk/index.htm]).

Ten clones were selected randomly from the primary L3 library. Inserts from three of these clones encoded proteins similar to PtCPR-1. One clone showed 99% nucleotide identity to the 5' end of *PtCpr-1*, differing by three nucleotides encoding three nonsynonymous amino acid substitutions. The second clone showed 96% nucleotide identity at the 5' end, differing by 20 nucleotides, and encoded 11 nonsynonymous amino acid

substitutions. Both clones incorporated a glycine at position 29 (not shown). The complete sequence of a third clone was obtained with vector and internal primers (cpr-2-F, cpr-2-R, and cpr-Int-R; 5'-CAT CAC CGC AAT CGT AAC AG-3'). It encoded a protein with 54% similarity to PtCPR-1 but contained the cysteine, histidine, and asparagine residues of the catalytic triad, as well as the 13 invariant cysteine residues (Fig. 2). Based on BLAST similarity to cathepsin B cysteine proteases and *PtCpr-1*, the gene was designated *PtCpr-2*.

Cysteine proteases are encoded by multigene families in other parasitic helminths (16, 20). It is likely that the four *P. tenuis* genes described here are part of a larger family. In addition, nematode populations can be genetically diverse and the different sequences may represent alleles.

Amplification of double-stranded cDNA from life stages of *P. tenuis* with gene-specific primers demonstrated that *PtCpr-1* (Fig. 1B) and *PtCpr-2* (Fig. 1C) were upregulated in larval stages compared with adults (L3 > L1 > adult). Nucleotide sequences of *PtCpr-1* amplicons from all life stages were identical.

PtCPR-1 is detected in L3 somatic extracts and E/S products. In Western blot assays of L3 somatic extracts, antibodies raised against rPtCPR-1 bound two proteins with apparent molecular masses of 46 and 38 kDa (Fig. 3, lane a). However,

FIG. 3. Synthesis of PtCPR-1 is developmentally regulated. Western blots of somatic extracts and E/S products from *P. tenuis* L3 were incubated with antiserum against rPtCPR-1 (lanes a and c) or *rlacZ* (lanes b and d). Anti-rPtCPR-1 bound 46- and 38-kDa antigens in L3 somatic extracts (lane a) and a 46-kDa antigen in L3 E/S products (lane g). Anti-*rlacZ* failed to bind antigens in E/S or somatic extracts (lanes b, d, f, and k). Neither antiserum reacted specifically with proteins from L1 or adult *P. tenuis* (lanes c, d, e, and f).

only a 46-kDa protein was detected in L3 E/S products (Fig. 3, lane g). PtCPR-1 was not detected in Western blot assays of either adult or L1 proteins (Fig. 3, lanes c and e). Control antiserum generated against *rlacZ* failed to bind the 46- and 38-kDa antigens (Fig. 3, lanes b, d, f, and h). The predicted molecular masses of the proprotein and putative mature PtCPR-1 were 37.1 and 28.3 kDa. Glycans attached to two putative N-glycosylation sites (Fig. 2) would contribute additional mass (up to 2.6 kDa per site based on glycan data from adult parasites; S. M. Haslam, M. S. Duffy, J. A. Appleton, and A. Dell, unpublished data). Thus, the mass of the 46-kDa E/S protein is most consistent with that predicted for a fully glycosylated proprotein of PtCPR-1. In this context, it is noteworthy that glycosylation of the NST sequence (amino acids 91 to 93) in the proprotein may interfere with cleavage of the proregion (Fig. 2).

Alternatively, the two protein species detected in somatic extracts may be accounted for by the presence of other, cross-reactive, cysteine proteases. Sequence identities among the four cDNAs identified may allow for cross-reaction with antisera raised against any one of them.

Larvae induce cellular infiltrates and granuloma-like reactions in snails. Prominent cellular infiltrates surrounding larvae were evident in H&E-stained sections prepared from snails as early as 1 dpi (Fig. 4A). The infiltrate was composed of hemocytes and by 7 dpi was surrounded by an outer rim of flattened fibroblast-like cells (Fig. 4B). Cellular reactions progressed to granuloma-like structures (Fig. 4C) that degenerated as hemocytes were replaced by amorphous and granular debris late in infection (Fig. 4D).

FIG. 4. Infection by *P. tenuis* induces cellular infiltrates and granuloma-like structures in the headfoot of snails. Snail tissues were collected prior to infection or at various days postinfection. Tissue sections were stained with H&E. Cellular infiltrates were composed of hemocytes (A, 1 dpi). Fibroblast-like cells accumulated early in infection (B, 7 dpi). The cellular reactions progressed to granuloma-like structures (C, 30 dpi) that degenerated later in infection (D, 105 dpi). Amorphous debris and granular materials accumulated in surrounding host tissues of degenerating granulomas (D). Arrowheads in the inset (D) = amorphous and granular materials in the L3 intestine and in degenerating granulomas; ci = cellular infiltrate; l = larva; f = fibroblast-like cells; g = granuloma; scale bar = 50 μ m.

Detection of PtCPR-1 in situ in infected snails. Antiserum raised against rPtCPR-1 detected the native protein within larvae beginning at 2 dpi. PtCPR-1 was restricted to the intestine and luminal contents of developing larvae (Fig. 5A to D) and was reduced over time (Fig. 5E). The protein was limited to larval tissues until 7 dpi (Fig. 5A), when it became detectable in surrounding host tissues, most notably in the cytoplasm of host hemocytes (Fig. 5F). By 105 dpi, the protein was detected almost exclusively in surrounding, degenerating granuloma-like structures (Fig. 5E). Control antiserum generated against *rlacZ* failed to bind any antigens in the headfoot of either infected or uninfected snails. Neither antiserum bound hemocytes from uninfected snails or tissues from adult parasites or L1. These data confirmed the developmental regulation of PtCPR-1 synthesis observed in Western blot assays.

Loss of PtCPR-1 from emerged L3. Antibodies detected a reduced abundance of PtCPR-1 in Western blot assays of emerged L3 compared with tissue L3 collected at the same time (90 to 105 dpi; Fig. 6A). Of the two bands detected in the extract shown in Fig. 3, the faster-migrating species was present but produced a weaker signal in this experiment. Although PtCPR-1 was less abundant in L3 that were recovered by digestion later in infection (135 dpi), reduction was not of the magnitude observed in emerged L3 (data not shown). Antibodies against the ubiquitous PtAPI-1 protein confirmed that protein loading was equivalent for tissue and emerged L3 (Fig. 6B).

Emergence of L3 from gastropods. Emergence of L3 was monitored in two separate trials. The first trial was initiated at 105 dpi. In this experiment, 685 emerged L3 were recovered from 44 snails between 105 and 258 dpi (Fig. 7A). One L3 was

FIG. 5. PtCPR-1 is synthesized by larval stages within the snail intermediate host. Immunohistochemical staining of infected snail tissues with antiserum against rPtCPR-1 detected the protein within the intestines of larvae and in surrounding granulomas. PtCPR-1 was detected exclusively within larvae at 7 dpi (A) but was also detected in surrounding host tissues at 14 dpi (B). The protein was restricted to the intestine (B, 14 dpi; C, 21 dpi) and luminal contents of larvae (D, 30 dpi; E, 105 dpi). PtCPR-1 was lost from larvae with progression of infection and accumulated in surrounding granuloma-like structures (E). Hemocytes throughout the headfoot of infected snails showed cytoplasmic, particulate staining for PtCPR-1 (arrowheads; F). e = esophagus; i = intestine; g = granuloma. Scale bar in panel A = 50 μ m and applies to panels A to E. Scale bar in panel F = 20 μ m.

observed emerging from the headfoot of an infected snail into the mucus trail. Emerged L3 represented 29% (685/2,370) of the total number of larvae recovered; the remaining tissue L3 were recovered from snails by digestion. Peak emergence occurred on the first day of the trial (105 dpi; Fig. 7A).

In the second trial, collections were initiated at 19 dpi. Larvae first emerged at 32 dpi, and a total of 146 L3 were recovered from 30 snails over 274 days of collection (Fig. 7A). Emerged L3 represented 17% (146/879) of the larvae recovered. Peak emergence occurred at 94 dpi (Fig. 7A). Intensities of infection declined with time, although differences were not statistically significant ($P = 0.059$; Fig. 7B).

Emerged L3 were opalescent and free of adherent cuticle from previous molts and appeared morphologically identical. Conversely, tissue L3 obtained by digestion of the host had varied morphologies. Some were fully developed, while others retained adherent cuticles from previous molts and had granular materials within the intestine. The latter morphology indicated that larvae had not completed development (41). Motile larvae were considered viable. When held at 4°C, 80% of emerged L3 (53/67) survived 1 month and 23% (11/48) were alive after 6 months. A few larvae survived as long as 1 year.

FIG. 6. PtCPR-1 is reduced in L3 that emerged from their intermediate host. Western blot assays of extracts from tissue and emerged L3 collected 90 to 105 dpi were developed with antiserum against rPtCPR-1 or a protein synthesized by all life stages of *P. tenuis* (PtAPI-1). (A) Reduced abundance of PtCPR-1 (46 kDa) in emerged L3 compared with tissue L3. (B) Equivalent numbers of tissue or emerged L3 contained similar quantities of PtAPI-1 (33 kDa).

Ten emerged L3 survived desiccation for 24 h at room temperature. Five and four live L3 were recovered from lettuce stored at -20°C for 24 h and 7 days, respectively. Infectivity of emerged L3 was not assessed.

DISCUSSION

Cathepsin B is encoded by a single gene in humans and other mammals (7). In contrast, the genomes of parasitic helminths (16, 20) and the free-living nematode *C. elegans* (43) appear to encode a diversity of cathepsin B-like cysteine proteases. Our preliminary investigation of *P. tenuis* identified several distinct cathepsin B-like transcripts, a result that is compatible with multigenic organization or allelic diversification. Whereas PtCPR-2 bears all the features of an active protease, PtCPR-1 and other paralogous sequences lack the essential active-site cysteine for which this class of proteases is named (34). Such C29G cathepsin B mutants have not been described for other organisms. However, when generated by site-directed mutagenesis, C29G substitution inactivates cysteine proteases (5, 8). Although intrinsic activities have yet to

FIG. 7. *P. tenuis* L3 emerge from infected snails (*Triodopsis* sp.). (A) Emerged L3 were observed in two separate trials. Trial 1 was initiated at 105 dpi, and trial 2 was initiated at 19 dpi. Larvae first emerged at 32 dpi, with onset of peak emergence at 94 to 105 dpi. (B) Infection intensities in snails declined over time (trial 2), although differences were not statistically significant ($P = 0.059$).

be documented, PtCPR-1 appears to be a nonpeptidase homologue of cathepsin B.

Synthesis of PtCPR-1 was restricted to the intestine, and protein was detected in luminal contents of larval stages developing within the intermediate host. A similar distribution has been described in flukes, where cathepsins are found in the lumen and epithelial cells of the intestine (6, 10, 39). Cathepsin proprotein is packaged into secretory granules in the gastrophodermis for delivery to the gut lumen, where proteolytic cleavage generates mature enzyme (8). Helminth cathepsin B-like proteases are thought to be central to nutrient acquisition by adult worms (2); however, our observation of a stage-specific cathepsin B in L3 E/S is more compatible with a role in development or tissue penetration, as seen in juvenile flukes (44). A cathepsin L is essential for embryogenesis and gut development in *C. elegans* (15), but its precise function is not known. Loss of PtCPR-1 from emerged *P. tenuis* L3 and its absence from adult worms and L1 are consistent with a primary role within the intermediate host. Although the specific functions of PtCPR-1 and PtCPR-2 have not been defined, either protein is positioned to influence intestinal development or tissue penetration. If the protein functions exclusively in intestinal development, its accumulation in snail tissue may result from excretion of excess protein. Alternatively, the protein may have a dual function such that the excreted protein may promote

tissue penetration and parasite release from the intermediate host. Should the low level of protein present in emerged larvae be sufficient, cathepsin B-like cysteine proteases may aid colonization of the vertebrate host (11, 12).

This is the first description of the emergence of *P. tenuis* L3 from its intermediate host. Emergence has been observed in other metastrongyloid nematode infections (4, 24) and occurs independently of variations in infection intensity or host species (24). Larval emergence has not been considered previously in *P. tenuis* transmission but may account for incongruent epidemiologic data. In areas with a high prevalence of infection, most white-tailed deer acquire *P. tenuis* during their first year of life (40). In contrast, the prevalence and intensity of L3 in naturally infected gastropods are generally very low (27). Although not conclusive, our results suggest that emergence contributes to the low prevalence and infection intensity observed in natural settings. Infectivity of emerged larvae was not assessed in our study but has been confirmed in related nematode species (17, 22, 23). Emerged *P. tenuis* L3 survived desiccation, freezing, and extended periods outside the host. Thus, emergence of L3 is likely to be an adaptation that promotes transmission of *P. tenuis* to susceptible vertebrate hosts.

The cellular events associated with the emergence of other metastrongyloid larvae (9, 18, 37) are similar to our observations of *P. tenuis*. The granuloma architecture around *P. tenuis* L3 degenerated and cellular infiltrates was replaced by granular debris by 90 to 105 dpi, corresponding to descriptions of necrotic hemocytes surrounding *Angiostrongylus cantonensis* L3 late in infection (45). It has been suggested that muscular contractions in the gastropod headfoot facilitate mechanical disruption of encapsulating granulomas and release of *Angiostrongylus costaricensis* L3 (9). Emergence may also be enhanced by the proximity of L3 to mucus ducts (32). Variation in the proximity of *P. tenuis* L3 to mucus ducts may explain the sporadic rather than synchronous nature of larval emergence from snails in our experiments.

The specific molecules in *P. tenuis* that mediate granuloma degeneration and larval emergence remain undefined. Degeneration of granuloma architecture coincided with accumulation of PtCPR-1 in tissue and its loss from L3. Although PtCPR-1 is putatively inactive, PtCPR-2 has an intact catalytic site and is produced by L3. There are likely to be additional active cathepsins, as reported for other helminths (16, 20), and proteases with similar sequences have been shown to cross-react with polyclonal antibodies (35). Unlike mammals, where cathepsin B activity is optimal at acid pH, helminth cathepsins are also active at neutral and alkaline pHs (38). This would allow nematode cysteine proteases that degrade extracellular matrix (36) to be active in gastropod host tissues. A functional protease could disrupt hemocyte adhesion and promote destruction of granulomas. In the case of *P. tenuis*, proteolysis might facilitate contact of larvae with mucus ducts, thereby enabling emergence.

Finally, a nonenzymatic role for PtCPR-1 is possible. A multigene family of inactivated cysteine proteases has been described for *Sarcoptes scabiei* (19). Novel roles for inactive proteases are recognized increasingly in invertebrate immune processes (25, 29, 46), including controlling proteolysis by active enzymes. This antagonistic role has been proposed for inactive serine protease homologues with comparable active-

site substitutions (serine → glycine; 25, 33). Since disruption of granuloma architecture coincides with its accumulation in host tissues, PtCPR-1 might interfere with immune down-modulation, thereby releasing or promoting an effective host immune response that would cause emergence (i.e., expulsion) of larvae.

In summary, we have found that synthesis of one or more cathepsin B-like proteases was developmentally regulated and restricted to larval stages residing within the intermediate host. The protein was limited to the intestine and luminal contents of developing larvae. Upon entry into snail tissues, larvae did not evade detection and were quickly encapsulated by infiltrating hemocytes. Loss of PtCPR-1 from L3 and its accumulation in the surrounding granuloma coincided with larval emergence from the snail intermediate host. Emerged larvae survived outside the host for prolonged periods, suggesting that vertebrate hosts would be infected by emerged L3 that contaminate vegetation. Our data implicate emergence as an adaptation that promotes transmission of *P. tenuis* by enhancing dissemination of the parasite in areas where susceptible vertebrate hosts graze.

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