Cathepsin B-like Cysteine Proteases Confer Intestinal Cysteine Protease Activity in *Haemonchus contortus*

Sankale Shompole‡ and Douglas P. Jasmer

From the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

Received for publication, August 11, 2000
Published, JBC Papers in Press, October 13, 2000, DOI 10.1074/jbc.M007321200

Cathepsin B-like cysteine protease genes (*cbl*) constitute large multigene families in parasitic and nonparasitic nematodes. Although expressed in the intestine of some nematodes, the biological and biochemical functions of the CBL proteins remain unresolved. Di- and tetra-oligopeptides were used as fluorogenic substrates and irreversible/competitive inhibitors to establish CBL functions in the intestine of the parasitic nematode *Haemonchus contortus*. Cysteine protease activity was detected against diverse substrates including the cathepsin B/L substrate FR, the caspase 1 substrate YVAD, the cathepsin B substrate RR, but not the CED-3 (caspase 3) substrate DEVD. The pH at which maximum activity was detected varied according to substrate and ranged from pH 5.0 to 7.0. Individual CBLs were affinity isolated using FA and YVAD substrates. pH influenced CBL affinity isolation in a substrate-specific manner that paralleled pH effects on individual substrates. N-terminal sequencing identified two isolated CBLs as *H. contortus* GCP-7 (33 kDa) and AC-4 (37 kDa). N termini of each began at a position consistent with preregion cleavage and protease activation. Isolation of the GCP-7 band by each peptide was preferentially inhibited when competed with a diazomethane-conjugated inhibitor, Z-FA-CCHN₂, demonstrating one functional difference among CBLs and among inhibitors. Substrate-based histological analysis placed CBLs on the intestinal microvilli. Data indicate that CBLs are responsible for cysteine protease activity described from *H. contortus* intestine. Results also support a role of CBLs in nutrient digestion.

This paper is available on line at http://www.jbc.org

Printed in U.S.A.
then, between CBLs and cathepsin B (16, 19). Such diversity might translate into CBL properties that are distinct from related mammalian enzymes. Specific properties have not been attributed to any individual *H. contortus* CBL, which has hinders more directed research on recombinant forms of these enzymes.

In this study, *H. contortus* intestinal CBLs were directly linked to known cysteine protease activity from this worm. Substrates for known cathepsin L, cathepsin B, caspase 1, and CED-3 (caspase 3) enzymes proved effective for dissecting distinct CBL activities. The results provide guidance to investigate these CBLs in relation to normal biological functions, functional diversity among individual CBLs and CBL contributions to anthelmintic-induced intestinal pathology.

**EXPERIMENTAL PROCEDURES**

**Materials—**Fluorogenic peptide substrates included Ac-YVAD.AMC, Ac-DEVAD.AMC, Z-PR.MNA, and Z-RR.MNA. Competitive peptide inhibitors included Ac-YVAD.CHO and Ac-DEVAD.CHO. Irreversible peptide inhibitors included Z-FAC.HN, Bi-YVAD.FMK, and Bi-FA.FMK. All of these substrates were obtained from Enzyme Systems Products (Dublin, CA).

**Parasite and Extracts—**Approximately 10,000 viable L3 larvae of a Beltsville isolate of *H. contortus* (21) were used to orally infect 4–8 month-old parasite-free lambs, which were killed 25–27 days after infection. Adult worms were harvested for dissection of intestines and for culture to obtain ESP (20).

**Protease Assays—**Intestines thawed from −80 °C were homogenized in PBS containing 1% Triton X-100. Protein concentration of lysates was determined by using the bicinchoninic acid protein assay reagent (Pierce). The extracts were stored in 200-μl aliquots at −80 °C. Protease activity was initially determined using the fluorogenic di- and tetrapeptide substrates, Z-FR.MNA, Z-RR.MNA, Ac-YVAD.AMC, and Ac-DEVAD.AMC at 50 μM concentrations. Reactions were performed in 1.5-ml volumes with 10 μl of intestinal extracts or ESP and incubated at 37 °C for 2 h. Samples were evaluated at 30-min intervals for 2 h and activity reported for the 2-h incubations. Worm samples without substrates served as negative control. Inhibition studies incorporated protease-specific class inhibitors including 1 μM phenylmethylsulfonyl fluoride, 10 μM E-64, 100 μM leupeptin, 1 μM pepstatin, 10 μM iodoacetate acid, 10 μM 1,10-phenanthroline, or 5 μM EDTA. In addition, substrate-based dipeptide (Z-FAC.HN) and tetrapeptide (Ac-YVAD.CHO, Ac-YVAD.FMK) protease inhibitors were used.

**pH Profiles—**The pH activity profiles were established using the following buffers: 100 mM citrate phosphate (pH 3.0–7.0) or 100 mM phosphate (pH 8.0) and 50 mM glycine-NaOH (pH 9.0) containing 10 mM DTT. Classic caspase activity was evaluated under conditions of 10 mM DTT, 31.25% CHAPS, 0.1% SDS, and 13.125% N-acetyl-L-cysteine. Immunolabeling of the liberation fluorescent groups, AMC and MNA, was monitored with a fluorescence spectrophotometer (MFP 42A, Perkin Elmer Life Sciences) using excitation and emission wavelengths of 380 and 460 nm (AMC), respectively, or 340 and 425 nm (MNA), respectively. Specific activity was determined in picomoles of AMC or MNA liberated per minute per microgram of protein at 37 °C. A standard curve produced with free AMC or MNA was used for these measurements.

**Affinity Isolation of Proteases—**Enzyme reactions were done by incubating 64 μg of intestinal extract with biotinylated irreversible substrate inhibitors Bi-YVAD.FMK or Bi-FA.FMK (5 μM) in 0.1 M citrate-phosphate buffer, pH 5.0, containing 10 mM DTT. The reaction mixtures were incubated for 15 min at 37 °C. Following this incubation, a mixture of protease inhibitors (100 μM leupeptin, 1 μM pepstatin, 10 μM 1,10-phenanthroline, and 10 μM E-64) was added to the solution. The mixture was adjusted to 0.2% SDS and incubated for an additional 15 min at room temperature with gentle agitation. This solution was used for affinity isolation procedures described below. Inhibition reactions were run by incubating the intestinal extracts with nonbiotinylated irreversible inhibitors, Ac-YVAD.FMK (5 μM), Z-FAC.HN (5 μM), or E-64 (10 μM) for 5 min (37 °C) prior to incubation with biotinylated substrate inhibitors.

Proteases conjugated to inhibitors were isolated by incubation with 100 μl of streptavidin-agarose beads (Sigma, St Louis, MO.) for 2 h at room temperature with gentle rotation. Bound beads were washed 5 times with 0.1 M glycerine and 1% SDS (pH 8.0). The streptavidin-agarose beads were boiled in 3X-sample buffer for 15 min. Eluted proteins were fractionated on 7.5–17.5% gradient SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described previously (22). The membranes were blocked overnight at 4 °C in PBS, Tween 20 (0.1%), and nonfat dry milk (5%). Membranes were incubated with streptavidin-agaroseshaped peroxidase conjugated (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:1000 in blocking buffer. Bound streptavidin was visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Amersham Place, United Kingdom). The M, of stained proteins was estimated using prestained Rainbow molecular mass standards ranging from 14.3 to 200 kDa (Amersham Pharmacia Biotech).

**N-terminal Amino Acid Sequencing—**Proteases were isolated using Bi-YVAD.FMK (pH 5.0) or Bi-FA.FMK (pH 5.0 and 7.0). Affinity isolation procedures were optimized in standard assays by titrating the following components: peptide inhibitors, DTT, streptavidin-agarose beads, and affinity isolation buffer. The peptide digest was then scaled up 50-fold to produce an estimated 450 pmol of proteins. Isolated proteases were fractionated on 7.5–17.5% gradient SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, MA). Transblotted proteins were visualized by staining with 0.1% Coomassie Blue for 10 min, followed by rapid destaining. Destained bands were excised and their N-terminal amino acid sequence determined using an Applied Biosystems 494/HT Procise sequencing system (University of Texas Medical Branch, Protein Chemistry Laboratory, Galveston, TX).

**Cytolocalization of *H. contortus* Intestinal Proteases—**Unstained intestinal proteases were determined using the bionitlated peptide inhibitors Bi-YVAD.FMK and Bi-FA.FMK. Whole worms were permeabilized in 4°C compounds (0.1% Triton X-100), fixed in 4°C, and rapidly frozen in liquid nitrogen. Five micrometer tissue sections were cut using a cryostate, attached to ProbeOn® Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and air-dried for 30 min. Tissue sections were fixed in 30% methanol, 60% acetone, and 10% distilled H2O for 30 s at room temperature. Subsequently, the tissues were rinsed in PBS and incubated at 37 °C for 15 min in 0.1 μM citrate-phosphate pH 5.0 buffer, 10 mM DTT, containing 5 μl Bi-YVAD.FMK or 5 μl Bi-FA.FMK. Inhibitor control tissue sections were pre-incubated with nonbiotinylated protease inhibitors or 5 μl E-64 for 15 min at 37 °C, followed by addition of bionitlated inhibitors. Negative control tissues were incubated without the peptide. The slides were then washed twice, for 5 min each, in wash buffer and blocked for 30 min at room temperature using PBS, Tween 20 (0.1%), and 10% normal sheep serum. Endogenous peroxidase inhibitor was included in reactions (Peroxidase Suppressor, ImmunoPure®; Pierce). Bound peptides were detected using streptavidin-agaroseshaped peroxidase conjugate diluted 1:500 in blocking buffer. Streptavidin and biotin complexes were visualized using the ImmunoPure Metal Enhanced DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate kit (Pierce).

**RESULTS**

**Peptide Substrate Recognition in *H. contortus* Intestine and ESP—**Protease activity was evaluated in intestinal extracts and ESP using four peptide substrates with the following sequences (designations): FR (cathepsins B/L), RR (cathepsin B), YVAD (caspase 1), and DEVD (caspase 3/CED-3). Initial interest in evaluating caspase substrates arose from the DNA fragmentation that can be induced by fenbendazole in intestinal cell nuclei of this parasite (10). Preliminary experiments at pH 5 demonstrated enzymatic activity against each substrate, with the exception of DEVD (Fig. 2A), but was further evaluated. Optimal pH for activity varied according to the substrate used. For example, activity against the YVAD substrate was restricted predominately to acidic pH, while activity against the RR substrate was highest around pH 6.0, and relatively high activity against the FR substrate occurred from pH 4.5 to 7.5 (Fig. 2, A–C). No activity against the DEVD substrate was detected.
under any of the described pH conditions, including preferred caspase conditions (data not shown). Significant activity against the RR substrate was not expected in *H. contortus* intestine based on a previous report (18). In addition, the preferential activity against YVAD under acidic conditions is inconsistent for caspases, which have neutral pH requirements (11).

Inhibitory effects of leupeptin and E-64 indicated that the majority of the activities against FR, RR, and YVAD substrates from intestine and ESP are due to cysteine proteases (Table I). Some variation on inhibition was observed among substrates with iodoacetic acid. Similar inhibitory results were obtained for FR and RR substrates at pH 5.0–6.0 (data not shown). Although 1,10-phenanthroline had moderate inhibitory effect on activities (57–87%), cysteine protease inhibition by 1,10-phenanthroline may be ascribed to a possible metal ion chelate. Although the relatively low inhibitory effect of EDTA supports this possibility, activity contributed by noncysteine proteases cannot be completely ruled out by these experiments. In contrast to the relative insensitivity of caspases to E-64 and leupeptin, intestinal activity against the YVAD substrate was ablated by both inhibitors. This result and observations on pH requirements suggested that activity against the YVAD substrate is conferred by noncaspase cysteine protease activity.

**Cross-inhibition of Protease Activity Using Peptide Substrates**—The possibility that related cysteine proteases confer activity against each of the substrates was evaluated by cross-inhibition experiments. Fluorogenic peptide substrates and peptide-based irreversible inhibitors were used in these experiments. Since no inhibitor construct was available for the RR substrate, experiments focused on the YVAD- and FR-based inhibitors. Additionally, a FA substrate modified by diazomethane (CHN$_2$) was previously shown to inhibit *H. contortus* cysteine protease activity (20) and was used here. Activity against each substrate was inhibited at pH 5.0 by the irreversible inhibitors Ac-YVAD.FMK and Z-FA.CHN$_2$ (Fig. 3). The inhibitor Ac-YVAD.CHO had similar effects on both YVAD and FR hydrolysis (data not shown). The high level of cross-inhibition suggested that a major portion of the activity against all substrates resided in the same enzymes at pH 5.0.

In contrast to YVAD, hydrolysis of the FR substrate was relatively high at pH 7.0. However, this activity was inhibited by Z-FA.CHN$_2$ (92%), Ac-YVAD.FMK (94%), and Ac-YVAD.CHO (78%). This result suggests that pH 7.0 inhibited protease binding by YVAD to a much lesser extent than protease hydrolysis of this substrate, which will be discussed below.

Nevertheless, the higher level of inhibition by the irreversible inhibitor Ac-YVAD.FMK compared with Ac-YVAD.CHO may indicate low level hydrolysis of this substrate at pH 7.0.

**Affinity Isolation of Cysteine Proteases**—Biotinylated peptide substrates modified by a fluoromethylketone reactive group were used to isolate, characterize, and identify proteases that hydrolyze the peptide substrates described. Use of Bt-YVAD.FMK led to isolation of multiple prominent bands ranging from 29 to 37 kDa (Fig. 4A), with a 42-kDa band that was weakly visible (lane 2). A 33-kDa band was most prominent in...
controls. 

respectively (Fig. 3), proteases from the 33-kDa band may be responsible for hydrolyzing these substrates. Alternatively, Z-FA.CHN2 could function in fluorogenic assays as a competitive inhibitor against proteases that are otherwise resistant to irreversible binding by this inhibitor. 

In intestinal extracts, CBLs are modified by the periodate-sensitive determinant that CBLs are recognized by mAb 42/10.6.1. This determinant occurs on a multitude of membrane/secreted/excreted proteins from H. contortus (14). Immunoblot analysis showed that most, if not all, proteins affinity-purified by Bt-YVAD.FMK and Bt-FA.FMK are modified by the mAb 42/10.6.1 determinant. In addition, the restricted size range of isolated proteins, compared with whole intestinal lysates, demonstrated a high level of purity in these preparations (Fig. 4B).

Table 1

| Class Inhibitor | Substratesa | YVAD | FR | RR |
|----------------|-------------|------|----|----|
| Cysteine       |             |      |    |    |
| Leupeptin      | 98.0       | 100  | 100| 100|
| E-64           | 99.0       | 100  | 100| 100|
| IAA           | 78.0       | 82.0 | 100| 100|
| Metallic      |             |      |    |    |
| 1,10-Phen     | 57.0       | 87.0 | 80.0| 1.8|
| EDTA          | 51.2       | 7.2  | 38.1| 5.5|
| Aspartic      |             |      |    |    |
| Pepstatin     | 1.7        | 0.0  | 10.7| 3.6|
| Serine        | 0.0        | 3.9  | 9.5 | 2.1|

a Fluorogenic peptide substrates Z-FR.MNA, Z-RR.MNA, and Ac-YVAD.AMC.

b Percentage of inhibition (S.D.) (n = 3), compared to non-inhibited controls.

c Metalloprotease.

d 1,10-Phenanthroline.

e Phenylmethylsulfonyl fluoride.

Substrate and pH-dependent Intestinal Protease Isolation—pH effects were evaluated on affinity isolation at pH 5.0, affinity isolated, electrophoretically fractionated, and detected (see “Experimental Procedures”). Samples for competition inhibition assays were pre-incubated with nonbiotinylated peptide inhibitors identified below the panel. Arrows indicate two prominent bands identified at 33 and 37 kDa. B, immunoblot of intestinal proteins purified with Bt-YVAD.FMK (lane 1), or from whole worm extracts (lanes 3), using mAb 42/10.6.1 (2 μg/ml). Isolated proteins from 64 μg of extracts or 25 μg of whole intestine were used. No reactivity was observed on a replicate blot using an isotype control mAb (IgG2a). Arrows point to the 33- and 37-kDa proteases.

Intestinal Cysteine Proteases in H. contortus

Fig. 3. Substrate-based cross-inhibition of intestinal protease activities. Intestinal extracts (10 μg) were pre-incubated for 5 min with peptide substrate inhibitors Ac-YVAD.FMK or Z-FA.CHN2 (50 μM) at pH 5.0 and then incubated with fluorogenic substrates Ac-YVAD.AMC, Z-FR.MNA, or Z-RR.MNA at 37 °C for 2 h. Results were expressed as mean percentage of inhibition of activity compared with reactions with no inhibitors. Bars represent standard deviations of triplicate assays.

Fig. 4. Affinity isolation of H. contortus intestinal proteases with biotinylated peptide inhibitors. A, intestinal lysates (64 μg) were incubated without Bt-YVAD.FMK (lane 1), with Bt-YVAD.FMK (lanes 2–5), or with Bt-FA.FMK (lanes 6–9) at pH 5.0, affinity isolated, electrophoretically fractionated, and detected (see “Experimental Procedures”). Samples for competition inhibition assays were pre-incubated with nonbiotinylated peptide inhibitors identified below the panel. Arrows indicate two prominent bands identified at 33 and 37 kDa. B, immunoblot of intestinal proteins purified with Bt-YVAD.FMK (lane 1), or from whole worm extracts (lanes 3), using mAb 42/10.6.1 (2 μg/ml). Isolated proteins from 64 μg of extracts or 25 μg of whole intestine were used. No reactivity was observed on a replicate blot using an isotype control mAb (IgG2a). Arrows point to the 33- and 37-kDa proteases.
Intestinal Cysteine Proteases in H. contortus

sensitive detection method. A size range of proteins was isolated from ESP by each substrate that paralleled those found in intestine (Fig. 5). The proteins isolated behaved in a substrate- and pH-dependent manner that paralleled intestinal proteins. The results also establish that the isolated proteins are modified by the mAb determinant. Collectively, results agree with a previous report (18) that the ESP proteins most likely represent intestinal cysteine proteases excreted from the parasite.

Identification of CBLs from Affinity Isolated Proteins—N-terminal sequencing was done on two isolated intestinal protein bands. The 33-kDa band was chosen based on evidence of activity against both YVAD and FA substrates. The 37-kDa band was chosen due to the relative resolution of this band and the resistance of the band to inhibition by Z-FA.CHN2. N-terminal sequences were obtained for bands isolated at pH 5.0 (FA, YVAD) and 7.0 (FA). Current data bases contain an estimated 11 published H. contortus CBL sequences (25) and ex-pressed sequence tags for an additional 11 unique CBLs. Two N-terminal sequences from each band had greatest similarity with H. contortus CBLs (Fig. 6). The sequences began at a uniform site consistent for pro-region cleavage, which is expected for the active proteases. Each sequence (20–24 residues) of the 37-kDa protein band isolated by each inhibitor, regardless of pH, exactly matched the H. contortus CBL AC-4 (Fig. 6). The residue corresponding to a single predicted cysteine residue was ambiguous in the protein sequences. The AC-4 sequence has multiple amino acids that are distinct from other CBLs, especially from consensus residues 11–17, providing a high degree of confidence in this identification.

Of the sequences from the 33-kDa band (10–17 residues), three exactly matched predicted amino acid sequences of the H. contortus CBL GCP-7 and an EST sequence (Hgcls1H4), which appears to represent GCP-7. These sequences were obtained from bands isolated by both the FA and YVAD affinity probes. The 33-kDa band obtained by FA isolation at pH 7.0 produced two alternative sequences due to heterogeneity (D/E) at the first residue (see fa7a and fa7b). The heterogeneity is likely to reflect more than one protease in this band. However, the dominant sequence clearly matches GCP-7 and the sequence is of sufficient length to instill a high degree of confidence in this identification.

Cytolocalization of Intestinal Proteases—Cytolocalization of the intestinal CBLs identified was determined with the biotinylated peptide inhibitors. In parallel with affinity isolation results, Bt-YVAD.FMK proved more sensitive in histological assays than Bt-FA.FMK (Table II). In sections of whole worms, Bt-YVAD.FMK binding was found only at the microvillar surface of the worm intestine (Fig. 7A). E-64 completely inhibited substrate binding to microvilli (Fig. 7B). Some background peroxidase activity localized to intestinal cytoplasm (Fig. 7C), but did not extend to microvilli. In other experiments, Z-FA.CHN2 and Ac-YVAD.FMK completely inhibited binding of the biotinylated peptides to intestinal proteases in the H. contortus intestines (Table II). Experiments with Bt-FA.FMK produced weaker staining on microvilli that could be inhibited by E-64 and Z-FA.CHN2 (data not shown). From these results,
we conclude that active intestinal CBLs localize to gut microvilli in vivo.

**DISCUSSION**

Our results extend previous observations in which intestinal cysteine protease activity of *H. contortus* was shown to be capable of hydrolyzing a FR substrate (18). Those experiments also used affinity labeling with Bt-FA.FMK (18) to identify four protein bands (30, 34, 37, and 41 kDa) from ESP. We isolated intestinal proteases of similar size with the same reagent and showed that two of these proteins are CBLs with identity to *H. contortus* GCP-7 (33 kDa) and AC-4 (37 kDa). Specificity of these CBLs was extended to include the YVAD substrate. Although each band may contain multiple proteases, our results provide confidence in identification of the predominant CBLs in these bands. We anticipate that proteins isolated in other bands also represent distinct CBLs.

In contrast to previous results (18), significant activity was detected against a cathepsin B substrate (RR) that was inhibited by FA and YVAD inhibitor analogues. The result suggests that CBLs are responsible for this activity, also. Reasons for the discrepancy are unclear, since similar substrates and conditions were employed in both investigations. Our result is curious, since many *H. contortus* CBL sequences lack a glutamate corresponding to position 245 in cathepsin B, which is required for similar activity in cathepsin B (16, 24). However, the CBL HmCP6 has a glutamate at this position (7) and other unidentified CBLs might also. Further, we cannot exclude that different laboratory isolates of unidentified CBLs might also be important that acidic residues (aspartate or glutamate) immediately precede the predicted proregion cleavage site for AC-3, AC-4, and AC-4. This position would represent the P1 position of CBLs that otherwise had catalytic activity against the FR substrate. Therefore, this effect may reflect displacement of the YVAD substrate in the binding pocket due to a pH influence on the CBL or substrate. For instance, a pH-dependent specificity switch occurs in cruzain, a *Trypanosoma cruzi* cathepsin L-like enzyme that can bind both hydrophobic and basic residues at different pH environments (27). Insufficient information is currently available for comments regarding the protease. However, the P1 aspartate in YVAD is the most likely substrate residue to be altered in charge at pH 7.0, which may account for the inhibition observed. Caspase substrate inhibitors YVAD.FMK and VAD.FMK have been shown to inhibit cathepsin B (26), but parallel observations on pH-dependent substrate binding were not reported for this protease. Our results raise the possibility that protonation of the P1 aspartate is important for caspase substrate interactions with cathepsin B.

Regarding the significance of YVAD substrate hydrolysis, it may be important that acidic residues (aspartate or glutamate) immediately precede the predicted proregion cleavage site for HmCP6, AC-3, and AC-4. This position would represent the P1 site for CBLs. Since cathepsin B proregion cleavage is autocatalytic (28, 29), there may be a biological need for at least a subset of CBLs to hydrolyze substrates with P1 acidic residues.

In contrast to fluoromethyl ketone inhibitors, the diazomethane derivative of the FA substrate preferentially inhibited affinity isolation of the GCP-7 protease band. The fluoromethylketone derivative is more specific to active site cysteines than diazomethane (27). However, this property alone should not discriminate among individual proteases, whereas structural differences among CBLs might. CBL sequence diversity in nematodes has stimulated much speculation regarding the functional significance of this diversity (3, 4, 11, 16). The preferential inhibition of the GCP-7 band provides the first direct evidence of functional differences among *H. contortus* CBLs. AC-4 and GCP-7 sequences are quite divergent and were associated with distinct CBL clades from *H. contortus* (16). Notable differences in predicted S'2 binding sites distinguished members of these clades (16). In this context, differences such as that indicated by Z-FA.CHN2 are not unexpected. This discriminating property of the diazomethane inhibitor might have value for characterizing recombinant CBLs and dissecting CBL properties in vivo. Alternatively, this inhibitor would appear to have limitations for use as a general irreversible inhibitor of CBL functions in vivo.

One objective of the research was to establish definitions and approaches that would support research on CBLs in a biological context. For instance, CBLs have been immunolocalized to *H. contortus* microvilli (7, 8). Each of the affinity isolated CBLs...
evaluated had N termini consistent with pro-region cleavage and protease activation. Hence, CBLs detected on microvilli by affinity probes are most likely active enzymes. These and other results (7, 8, 18) support that CBLs have a major role in nutrient digestion by *H. contortus*. Further, 17% of expressed sequence tags from *H. contortus* intestine were cbl genes, which dominated all other intestinal genes/gene families found. 2 These results indicate a prominent role for CBLs in the biology of *H. contortus*. In addition, fenbendazole treatment cause secreted intestinal proteins modified by the mAb 42/10.6.1 determinant to become dispersed in the cytoplasm of *H. contortus* intestinal cells that undergo degeneration (10). The dispersed proteins are likely to include CBLs, since CBLs are expressed in intestinal cells that undergo degeneration (10), and, as shown here, are modified by the mAb 42/10.6.1 determinant. With the broad pH requirements observed, CBLs become potential candidates for mediating intestinal pathology induced by the anthelmintic fenbendazole. Finally, CBL activity can be distinguished from classic caspase activity, which may be important for elucidating cellular mechanisms of fenbendazole-induced DNA fragmentation (10).

**Acknowledgments**—We appreciate the excellent technical support of Xiaoya Cheng and Stewart G. Bohnet in this work. Protein sequencing was done with support from the Protein Chemistry Laboratory at the University of Texas Medical Branch Educational Cancer Center (Galveston, TX) and the University of Texas Medical Branch NIEHS center grant.

**REFERENCES**

1. Pratt, D., Armes, I. G., Hageman, R., Reynolds, V., Boisvenue, R. J., and Cox, G. N. (1992) *Mol. Biochem. Parasitol.* 51, 209–218
2. Prociv, P., and Brindley, P. J. (1995) *Trop. Med. Parasitol.* 46, 119–122
3. Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Showkenne, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K., Thierrymieg, J., and Sulston, J. (1992) *Nat. Genet.* 1, 114–123
4. Larminie, C. G., and Johnstone, I. L. (1996) *DNA Cell Biol.* 15, 75–82
5. Brindley, P. J., Kalinna, B. H., Dalton, J. P., Day, S. R., Wong, J. Y., Smythe, M. L., and McManus, D. P. (1997) *Mol. Biochem. Parasitol.* 89, 1–9
6. Wasilewski, M. M., Lim, K. C., Phillips, J., and McKerrow, J. H. (1996) *Mol. Biochem. Parasitol.* 81, 179–189
7. Skuce, P. J., Redmond, D. L., Liddell, S., Stewart, E. M., Newlands, G. F., Smith, W. D., and Knox, D. P. (1999) *Parasitology* 119, 405–412
8. Knox, D. P., Smith, S. K., and Smith, W. D. (1999) *Parasite Immunol.* 21, 201–10
9. Smith, S. K., Pettit, D., Newlands, G. F. J., Redmond, D. L., Skuce, P. J., Knox, D. P., and Smith, W. D. (1999) *Parasite Immunol.* 21, 187–199
10. Jasmern, D. P., Yao, C., Rehman, A., and Johnson, S. (2000) *Mol. Biochem. Parasitol.* 105, 81–90
11. Alnemri, E. S. (1997) *J. Cell. Biochem.* 64, 3–42
12. Vaux, D. L., Wilhelm, S., and Hacker, G. (1997) *Mol. Cell. Biol.* 17, 6592–6597
13. Xue, D., Shaham, S., and Horvitz, H. R. (1996) *Genes Dev.* 10, 1073–1083
14. Jasmern, J. P., Peryman, L. E., Cender, G. A., Crow, S., and McGuire, T. C. (1999) *J. Immunol.* 161, 5450–5460
15. Rehman A., and Jasmern, D. P. (1998) *Mol. Biochem. Parasitol.* 97, 55–68
16. Rehman, A., and Jasmern, D. P. (1999) *Mol. Biochem. Parasitol.* 102, 297–310
17. Cox, G. N., Pratt, D., Hageman, R., and Boisvenue, R. J. (1990) *Mol. Biochem. Parasitol.* 41, 25–34
18. Rhoads, M. L., and Fetterer, R. H. (1995) *J. Parasitol.* 81, 505–512
19. Mustil, D., Zucic, D., Turk, D., et al. (1991) *EMBO J.* 10, 2321–2330
20. Karamu, F. N., Rubangire, F. R., McGuire, T. C., and Jasmern, D. P. (1993) *Exp. Parasitol.* 77, 362–371
21. Jasmern, D. P., and McGuire, T. C. (1991) *Infect. Immun.* 59, 4412
22. Towbin, H., and Gordon, H. (1984) *J. Immunol. Methods* 72, 313–340
23. Hughes, A. L. (1994) *Mol. Phylogenet. Evol.* 3, 310–321
24. Hasnain, S., Hiranma, T., Huber, C. B., Mason, P., and Mort, J. S. (1993) *J. Biol. Chem.* 268, 235–240
25. Schoetz, P., Dedreng, W., Huffel, S., Vandenabeele, P., and Beyaert, R. (1999) *FEBS Lett.* 442, 117–121
26. Shaw, E., Angliker, H., Rauber, P., Walker, B., and Wikstrom, P. (1986) *Biomed. Biochim. Acta* 45, 1397–1403
27. Gilmore, S. A., Craik, C. S., and Fletterick, R. J. (1997) *Protein Sci.* 6, 1603–1611
28. Hasnain, S., Hiranma, T., Tam, A., and Mort, J. S. (1992) *J. Biol. Chem.* 267, 4713–4721
29. Fox, T., Miguel, E., Mort, J. S., and Storer, A. C. (1992) *Biochemistry* 31, 12571–12576
